FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Identifying the cause of Oyster Oedema Disease (OOD) in pearl oysters (*Pinctada maxima*), and developing diagnostic tests for OOD

Priscila Goncalves[1], David Raftos[1], David Jones[1,a], Kelli Anderson[1,b], Brian Jones[2] & Michael Snow[3]

1st February 2017

FRDC Project No 2013/002

[1] Department of Biological Sciences, Macquarie University, NSW 2109, Australia; [2] Animal Health Laboratory, Ministry for Primary Industries, New Zealand; [3] Department of Fisheries, WA, Australia. [a] Current address: Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, QLD 4811, Australia; [b] Current address: Faculty of Science, Health and Education, University of the Sunshine Coast, QLD 4558, Australia.

MACQUARIE University

Ministry for Primary Industries
Manatū Ahu Matua

Government of Western Australia
Department of Fisheries

ISBN 978-0-646-96779-0

Identifying the cause of Oyster Oedema Disease (OOD) in pearl oysters (*Pinctada maxima*), and developing diagnostic tests for OOD

FRDC Project No 2013/002

2017

Ownership of Intellectual property rights

Creative Commons licence

Disclaimer

Researcher Contact Details

Address:	Department of Biological Sciences
	Macquarie University
	North Ryde NSW 2019
Phone:	02 9850 8402
Email:	david.raftos@mq.edu.au
Web:	www.mq.edu.au

FRDC Contact Details

Address:	25 Geils Court
	Deakin ACT 2600
Phone:	02 6285 0400
Fax:	02 6285 0499
Email:	frdc@frdc.com.au
Web:	www.frdc.com.au

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Figures

Figures

Tables

ACKNOWLEDGEMENTS

The authors are grateful for the contribution and continuous support of the following industry partners: Pearl Producers Association (PPA), Paspaley Pearls, Cygnet Bay Pearls, Clipper Pearls and Tenerife/Norwest Pearls. We also thank Dr. Ben Madin from AusVet - Animal Health Services Pty Ltd - and staff from the Kimberly Marine Research Station (KMRS; Cygnet Bay) for their advice and assistance.

EXECUTIVE SUMMARY

The goal of this project was to investigate the cause of oyster oedema disease (OOD) in Australian pearl oysters so that diagnostic tests and management practices for the disease can be developed. OOD has been associated with mortalities in some pearl oyster farming areas. However, the cause of these mortalities has remained unknown, hampering efforts to study the disease and develop effective control strategies. The project described in this report was conducted by researchers from Macquarie University, Fisheries Western Australia and the New Zealand Ministry for Primary Industries (Manatū Ahu Matua), working in collaboration with the Australian Pearl Producers Association and the Australian pearling industry. We compared OOD-affected oysters with healthy control oysters to identify any genetic material in the OOD-affected oysters that might come from an infectious agent such as a virus, bacteria or parasite. Our logic was, if OOD is caused by an infectious agent, genetic material (cDNA nucleotide sequences) from that infectious agent should be far more abundant in OOD-affected oysters than in healthy controls. That genetic material would act as a fingerprint for the disease and may provide information about its cause. We found clear differences between the nucleotide sequences present in oysters affected by OOD when compared to healthy controls. A number of nucleotide sequences were strongly associated with OOD and the abundance of some of these sequences was correlated with increasing mortality. None of these nucleotide sequences were closely related to any known infectious agents. However, the strong relationship between these sequences, OOD and mortality means that they may be very useful predictors of mortality. Their lack of resemblance to known infectious agents leaves open the possibility that OOD is not an infectious disease and may have some other cause.

BACKGROUND

In 2006, severe mortalities (up to 100%) were reported among pearl oyster production farms in the Exmouth Gulf, Western Australia. Only Australian silver-lipped pearl oysters (*Pinctada*

maxima) were affected, while other molluscs such as black-lipped pearl oysters (*Pinctada margaritifera*) remained healthy (Madin 2007). One early study indicated that mortality was due to an infectious disease. However, these results were contradicted by subsequent work and no obvious disease causing agent could be associated with the mortalities. Histology revealed no inflammatory response or obvious infectious agents, such as viruses, in tissue from oysters at high mortality sites. The only clinical sign that could be detected were subtle lesions involving tissue oedema (swelling) (Jones *et al.* 2010). Hence, the mortalities were ascribed to Oyster Oedema Disease (OOD). Sporadic high mortality events among farmed *P. maxima* continue to occur in northwest Australia. Some of these have been confirmed by histology to represent cases of OOD. However, there is still no conclusive evidence that OOD is caused by an infectious agent, or what that infectious agent might be.

AIMS AND OBJECTIVES

Given the lack of information about the cause of OOD, the aims of our project were to:

(i) identify pathogens that may be contributing to OOD;

(ii) establish their role in causing the disease;

(iii) develop a diagnostic test for the disease based on this knowledge.

These aims gave rise to the following specific objectives:

1. Identify the presence of any pathogens (as defined by their nucleotide sequences) associated with OOD-affected oysters using a next-generation sequencing approach;

2. Use quantitative real-time PCR to measure the prevalence of these nucleotide sequences in a broad range of oyster samples, including samples from a longitudinal sampling experiment;

3. Undertake a new electron microscopy study of tissue from OOD-cases to visually identify any potentially pathogenic organisms in the oyster tissue;

4. Work with the AAH Subprogram and industry to identify appropriate timing and planning for a workshop to focus on extension of results from this project, and any gaps in our knowledge.

METHODOLOGY

The project involved two sequential phases of research.

• *Phase 1:* The first phase was to identify nucleotide sequences associated with all potentially pathogenic microorganisms in OOD-affected oysters (relative to non-affected controls) using next-generation sequencing (NGS) (*Objective #1*).

• *Phase 2:* The second phase of the project was to test whether the abundance of any of the nucleotide sequences identified in *Phase 1* is directly correlated with the presence of OOD or increasing mortality, and so may be indicative of the disease (*Objective #2*). Fresh electron microscopy analyses to identify infectious agents also fell under this phase of the work (*Objective #3*).

This workflow led to a sequential process of identifying nucleotide sequences (contigs-of-interest) that seemed to be associated with OOD and then refining that list of contigs-of-interest by further analysing them against increasingly large numbers of confirmed OOD-cases, OOD-non-

cases (unaffected controls), and oysters suspected of suffering from OOD (OOD-suspected). The workflow is summarised by the schematic diagram shown in Figure 1.

RESULTS

Next-generation nucleotide sequencing identified clear and substantial differences between oysters affected by OOD when compared to healthy controls. Among these differences, we identified five nucleotide sequences that are strongly associated with OOD. These sequences were consistently found at higher abundance in OOD-cases relative to healthy controls collected across broad geographical and temporal ranges. Some combinations of these sequences identified up to 75% of OOD-cases, and none (or less than 10%) of non-case control oysters. The same combinations of sequences identified 20% to 60% of oysters from areas of high mortality that showed gross signs of OOD without histopathological evidence of oedema. The relative abundance of two sequences was also positively correlated with cumulative mortality in oyster samples collected from a longitudinal (time-course) sampling experiment. One of the sequences that was positively correlated with cumulative mortality came from our list of five nucleotide sequences that are strongly associated with OOD. The other fell just outside the very stringent criteria we used to identify sequences that delineated OOD-cases from non-cases. None of the sequences are closely related to known infectious agents, such as known viruses, bacteria or parasites. Extensive electron microscopy analysis also did not identify any viruses in tissue from OOD-cases, even though some intracellular bacteria were detected.

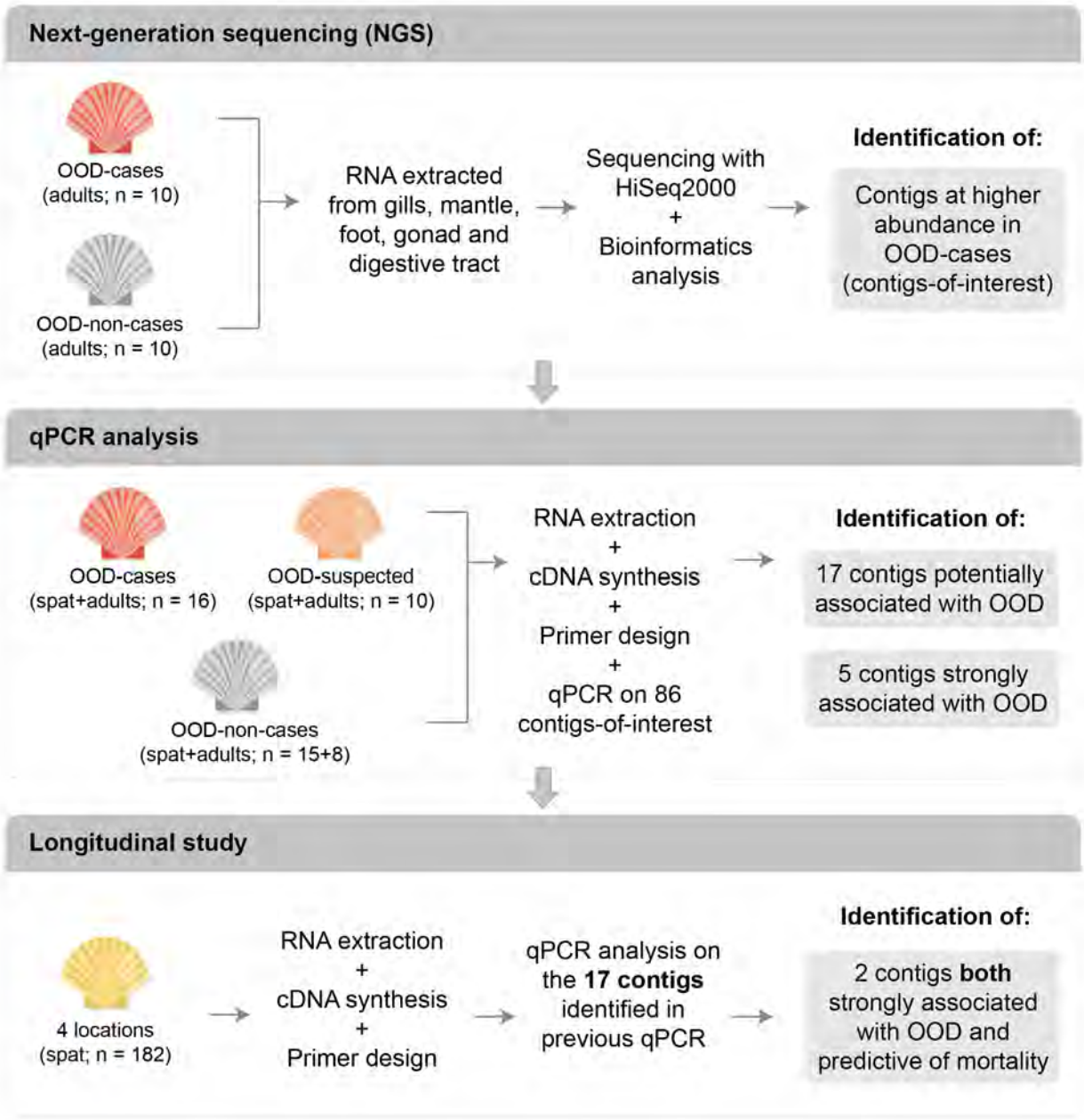

Figure 1. Schematic diagram of the sequential processes used to identify nucleotide sequences associated with OOD. OOD-cases (in red) were oysters collected from areas suffering high mortalities exhibiting both gross and histopathological signs of OOD (as defined in the case definition for the disease). OOD-suspected oysters (in orange) came from areas suffering high mortalities exhibiting gross signs of OOD, but lacked histopathological signs of oedema. OOD-non-cases (non-affected controls; in grey) were oysters from areas of low mortality that were lacking both gross and histopathological signs of OOD and were otherwise deemed by farmers as healthy. Oysters collected from the longitudinal sampling study (in yellow) were not classified into any of these three categories (*i.e.* OOD-cases, OOD-suspected and OOD-non-cases).

IMPLICATIONS FOR RELEVANT STAKEHOLDERS

Our analyses provide no evidence for a virus as the causative agent of OOD. We are confident that our next-generation sequencing (NGS) approach would have identified any causative infectious agent that was present at high abundance when indicative features of OOD (high mortality, gross signs and tissue oedema) were apparent in oysters. We have also limited the possibility of not identifying cryptic infectious agents (those that are restricted to a highly localised site of infection within oysters and gain their pathological effects systemically) by sampling a broad range of tissues within each oyster. However, it is possible that a pathological agent, such as a harmful alga, might remain external to the oyster and gain its effects through the release of a chemical toxin, which we would not have identified by NGS.

Despite this qualification, we have identified five contigs-of-interest that provide a clear delineation between OOD-case and non-case oysters, as well as identifying many oysters that were suspected of OOD (high mortality area and gross signs of disease) but did not show tissue oedema. We also identified one addition contig-of-interest that was strongly correlated with mortality in a longitudinal study and fell just outside our stringent criteria for delineation between OOD-case and non-case oysters. The samples that we tested were from a broad range of geographic locations and times, so the patterns of contig abundance that we identified are unlikely to reflect location- or time- specific differences in the normal microbiota of oysters. None of our final list of contigs-of-interest identified all OOD-case oysters, even though pairs of contigs identified up to 75% of OOD-cases and up to 60% of OOD-suspected oysters. Less than 100% identification was not unexpected, since some of the oysters in a sample group may not have been at a stage of disease where the pathological agent was readily detectable.

Our final shortlist of contigs-of-interest could reflect the presence of a single (previously uncharacterised) pathological agent. Alternatively, they may represent changes in oysters resulting

from environmental stress, abiotic disease and morbidity. Regardless, these contigs-of-interest are strongly indicative of OOD.

RECOMMENDATIONS

We recommend that our study be supplemented with a thorough analysis of the oyster genes that respond to OOD. We believe that patterns of response among oyster genes will reveal the underlying cause of OOD and will provide more effective and predictive diagnostic markers of the disease. In addition, the development of multigene arrays or other advanced analytical technologies based on the data from this project should be further explored as effective diagnostic tools for OOD. We also believe that analyses of factors external to oysters, such as environmental stressors and harmful algae, should be investigated.

KEYWORDS

Pinctada maxima, silver-lipped pearl oyster, Oyster Oedema Disease, next-generation sequencing, disease.

1. INTRODUCTION

1.1 Oyster Oedema Disease (OOD)

Pearl oyster farming is one of Australia's most successful aquaculture industries, with substantial scope for future growth. Recent data suggest that the pearling industry generates approximately AUD$60 million per year and provides significant employment opportunities in remote areas of Australia (Savage & Hobsbawn 2015). Despite its remarkable success, the viability of Australian *Pinctada maxima* pearling is compromised by a fatal disease. Oyster Oedema Disease (OOD) was first reported during October 2006 among silver-lipped pearl oysters (*P. maxima*) farmed in the Exmouth Gulf of Western Australia (WA) (Madin 2007). More than 2.8 million *P. maxima* died during the initial outbreak. Mortality rates of approximately 60% occurred amongst recently seeded oysters. Smaller oysters (shell length 2-10 cm) were the most susceptible (up to 90% mortality), but all oyster size classes were impacted (Humphrey & Barton 2009). Since 2006, OOD has limited the productivity of *P. maxima* farming in some affected locations, and the disease continues to threaten growing areas throughout WA and the Northern Territory (NT) (Jones 2012; Jones & Raftos 2012). This has substantially decreased the number of oysters available to produce pearls in affected areas.

There are few obvious signs of OOD before oysters die. Mild oedema and retraction of the mantle is evident, but histological examinations reveal no inflammatory response. Subtle lesions involving tissue oedema and oedematous separation of the epithelia from underlying tissues occur in affected oysters before death (Jones *et al.* 2010). However, the time taken for oysters to die after the initial appearance of oedema is so short (days) that these clinical features are of little value in providing early warning of disease.

The cause of OOD remains unknown, but some limited evidence suggests that it results from infection. During the initial outbreak in 2006, OOD mortalities spread between leases (lines) that were separated by several kilometres (Madin 2007) (B. Jones, personal communication; J.

Brown, Cygnet Bay Pearls, personal communication). Since then, observations by pearl farmers suggest that even within leases, OOD radiates outward from an initial focal point of mortality (J. Brown, personal communication). Other bivalve species in OOD impacted areas (including black-lipped pearl oysters, *Pinctada margaritifera*) are not affected by the disease. These patterns of disease are consistent with the spread of a host-specific infectious agent (pathogen). However, controlled laboratory cross-infection experiments have provided equivocal results (Humphrey & Barton 2009; Spiers & Bearham 2006), and histopathology has not identified a definitive pathogen associated with OOD (Jones *et al.* 2010).

The lack of information regarding the cause of OOD has substantial impacts on the pearling industry. Pearl farmers do not have sufficient information to establish effective management strategies that can control OOD and avoid major loses in production. Diagnostic methods to detect the onset of disease before catastrophic outbreaks occur represent an urgent need to secure the Australian pearling industry. Such tests could be used to identify disease-free broodstock for hatcheries, find areas where OOD is less prevalent, determine environmental factors that might be associated with the disease, and manage the translocation of OOD-affected oysters.

1.2 Next-generation sequencing as a tool to identify the cause of OOD

Given the lack of information on the cause of OOD, broad analytical approaches are required for the identification of any infectious agent(s) that might be associated with OOD. Next-generation sequencing (NGS) allows infectious agents (viruses, bacteria, protozoans, etc.) to be identified without any prior knowledge of the type of pathogen that might be involved in a disease, as is the case with OOD. This approach consists of large-scale sequencing of fragments from thousands of genes (and non-coding nucleotide sequences) rapidly and at relatively low cost (Barzon *et al.* 2013; Mardis 2008).

The logic behind using NGS for pathogen discovery is that, if large amounts of genetic material (DNA or RNA) from diseased animals are sequenced, the resulting data will include

nucleotide sequences from the pathogens infecting the animal. This is true even if the pathogen is present within the host at low concentrations. Pathogen sequences act as "nucleotide fingerprints". They can be searched against existing databases (GenBank or custom databases) that contain sequence data for many thousands of known bacteria, viruses and other infectious agents (Moore *et al.* 2011). This allows unknown infectious agents to be identified based on their similarities to known pathogens, in much the same way that the fingerprints of suspected criminals are automatically searched against vast crime databases. If pathogens associated with OOD are new, it is still possible that they will be sufficiently closely related to known infectious agents to be detected by this method (Løvoll *et al.* 2010). Even without a positive identification, sequences that accurately differentiate diseased and healthy animals can be extremely useful in terms of predictive diagnosis and the development of management practices to control mortalities.

Once differentially abundant sequences have been identified by NGS, their role in disease can be rigorously analysed using high throughput techniques, such as quantitative (q) PCR. This allows cause and effect models of disease to be tested, confirming the direct relationship between the presence or abundance of a particular sequence and the occurrence of disease. The same data can then be used to develop predictive diagnostic tests of disease and mortality.

NGS approaches have been extensively used to identify pathogens responsible for human diseases (e.g. Barzon *et al.* 2013, Barzon *et al.* 2011, Lecuit and Eloit 2015), and they are now being applied to diseases that affect aquaculture. For instance, Løvoll *et al.* (2010) used NGS to identify a novel totivirus as the pathogen causing cardiomyopathy syndrome (CMS). CMS is a severe disease that affects farmed Atlantic salmon and often appears without prior clinical signs. Once the totivirus had been identified by NGS, its role in disease was confirmed by qPCR. qPCR showed that the totivirus was detected in 100% of samples from CMS-affected fish, but not in non-affected controls. This process is now a recognised application of NGS. Sequencing datasets can be mined for the presence of sequences for viral, bacterial and eukaryotic pathogens, confirming infection by unknown pathogens or discovering new ones (Gómez-Chiarri *et al.* 2015).

2. AIMS AND OBJECTIVES

The management of OOD by the Australian pearl oyster industry is hindered by the lack of an identified causative agent for the disease and an understanding of the disease process. This makes it difficult to gauge factors associated with disease susceptibility and to predict the onset of mortality events. Hence, the aims of the current project were:

(i) to identify any pathogens that may be contributing to OOD;

(ii) to establish their role in causing the disease;

(iii) to develop a diagnostic test for the disease based on this knowledge.

These aims gave rise to the following specific objectives[1]:

1. Identify the presence of any pathogens (as defined by their nucleotide sequences) associated with OOD-affected oysters using a next-generation sequencing approach;
2. Use quantitative real-time PCR to measure the prevalence of these nucleotide sequences in a broad range of oyster samples, including samples from a longitudinal sampling experiment;
3. Undertake a new electron microscopy study of tissue from OOD-cases to identify any potentially pathogenic organisms in the oyster's tissue;
4. Work with the AAH Subprogram and industry to identify appropriate timing and planning for a workshop to focus on extension of results from this project, and any gaps in our knowledge.

[1] These specific objectives were modified from those listed in the original funding proposal. They represent the objectives listed in the Revised Research Plan, which evolved as the project progressed.

3. RESEARCH PLAN

The central rationale for this project is that any infectious pathogen causing disease can be identified in its host by searching for the pathogen's RNA sequences. Apart from prions, we are not aware of any pathogen that does not produce RNA during the proliferative phase of its life cycle. Pathogens causing disease states are likely to be at high abundance in their hosts and to be represented by substantial quantities of RNA (qualifications of these assumptions are discussed in *Conclusions, Perspectives and Implications*). Hence, pathogens associated with an uncharacterised disease can be identified by comparing the abundance of their RNAs between diseased and healthy hosts. The causative agent might also be identified (named) by comparing its RNA sequences to those for known pathogens in publically available sequence databases.

Our project took this approach to identify causative agents that might be associated with OOD. To meet its objectives, the project was divided into two sequential phases of research based on different technologies (see Figure 1).

• *Phase 1:* Next-generation sequencing

The first phase of the project was to identify nucleotide sequences associated with all potentially pathogenic microorganisms in OOD-affected oysters (OOD-cases) relative to non-affected controls (OOD-non-cases) using next-generation sequencing (NGS) (*Objective #1*). NGS is a relatively low throughput technology in terms of number of samples that can be analysed (at an affordable cost), but provides vast amounts of sequence data per sample. Hence, in this first phase of the project, we used tissue samples from just 20 pearl oysters – 10 OOD-cases and 10 non-cases. cDNA (derived from both total RNA and mRNA) from four individual oysters from each group plus a pool of all ten oysters from each group were subjected to NGS. The resulting sequence reads from each treatment (OOD-cases vs. non-cases) were assembled into larger

contiguous sequences (contigs) so that the contigs present in OOD-cases could be compared to those in non-case oysters.

• *Phase 2:* qPCR and electron microscopy

The second phase of the project was to further test nucleotide sequences (contigs) that were found in *Phase 1* to differ significantly in abundance between the small numbers of OOD-cases and non-cases used for the NGS analysis. The goal was to determine whether any of the differential sequences identified by NGS was directly correlated with the presence of OOD and/or increasing mortality, and so might be indicative of the disease (*Objective #2*). This work used a high throughput technology (quantitative real-time polymerase chain reaction, qPCR) to screen far greater numbers of OOD-cases, non-cases and suspected cases than was possible in *Phase 1*. The oyster samples used in *Phase 2* came from opportunistic (one-off) sampling (49 oysters) when farmers reported unusually high mortalities, and from a time-course (longitudinal) sampling experiment (182 oysters) where oysters were sampled sequentially over a substantial period of time to capture an entire high mortality event. This mix of samples allowed us to test both the frequency with which contigs were present in OOD-cases and absent from non-cases, and to correlate contig abundance with increasing mortality. The samples used for qPCR came from a range of different geographic locations. In some cases, OOD-cases and non-cases came from the same locations. This enabled us to rule out simple geographic differences in the normal microbiota of oysters as a cause for differential contig abundance. During *Phase 2*, we also undertook fresh electron microscopy analyses to identify viruses and bacteria within the tissues of OOD-cases so that the presence of these potential pathogens could be compared to the results of NGS and qPCR analyses (*Objective #3*).

This workflow led to a sequential process of identifying nucleotide sequences (contigs-of-interest) that seemed to be associated with OOD and then refining that list of contigs-of-interest by

further analysing them against increasingly large numbers of OOD-cases, OOD-suspected oysters and OOD-non-cases. That process of elimination is summarised by the schematic diagram shown in Figure 1.

4. METHODOLOGY

4.1 Sample collection

This project was based on the opportunistic sampling of oysters and on an experimental longitudinal (time-course) study. Both were designed to provide samples from oysters that were likely to be affected by OOD (OOD-cases) as well as healthy oysters that were not affected by the disease (OOD-non-cases). The sampling effort spanned 10 years covering 5 locations (12 independent sampling events) in the Northern Territory (NT) and Western Australia (WA). It represents an intensive, collaborative effort by pearl farmers, pathologists and researchers.

4.1.1 Categorisation of samples based on the formal case definition of OOD

Samples were categorised into OOD-cases and non-cases based on the formal case definition of OOD as reported in the AusVet Animal Health Services Pty Ltd assessment of OOD prepared for the Western Australian Department of Fisheries by Dr. Ben Madin (2007) (see Box 1) and supplemented by Jones *et al.* (2010). This involved industry assessments of the condition of the areas (farms) that oysters were collected from (high vs. low mortality), the presence of gross signs (according to the case definition of OOD) and accompanying histopathological analyses (when available). Madin (2007) reported that OOD-cases present with gross signs of mantle retraction, swelling (oedema) of the body (especially the digestive tract) and reduced adductor muscle function (weak or delayed closure) (Fig. 2). Other organs appear healthy. At the histological level, apart from oedema, no inflammatory response and no other significant changes are evident (Jones *et al.* 2010; Madin 2007).

Box 1. Formal case definition for OOD.

Case definition:

Due to the non-specific signs in the dead or dying animals, the case definition for animals takes into account the condition on the farm at the time of mortalities.

Affected animals:

- Silver-lipped pearl oysters (*Pinctada maxima*) only
- Dead or dying, with
- (Grossly) mantle retraction, poor adductor muscle function and mild swelling, otherwise normal in appearance (Fig. 2), and/or
- (Histology) showing no signs of an inflammatory process or the presence of pathogens
- On a farm on which mortalities have exceeded normal mortalities for the developmental and management stage of the oyster.

Affected leases:

- Farms experiencing mortalities greater than expected in the age group of animals matching the animal case definition
- With no unusual mortalities in other species of animal
- With no known precipitating cause.

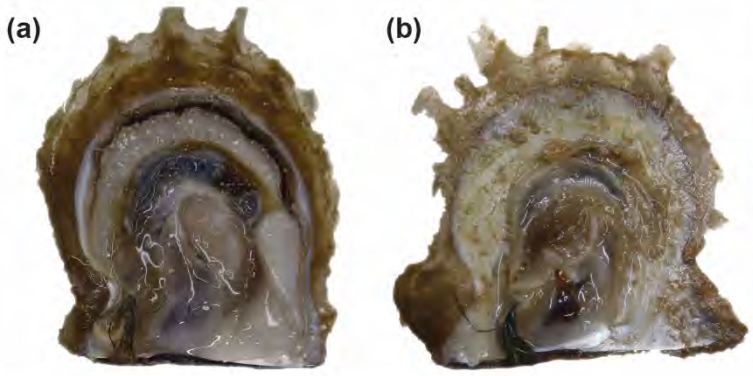

Figure 2. Examples of healthy **(a)** and OOD-affected **(b)** silver-lipped pearl oysters (*Pinctada maxima*). Sick oyster **(b)** shows mantle retraction, mild swelling and no evidence of recent growth, fitting the formal case definition for OOD.

Based on this case definition (Box 1),

OOD-cases: were collected from areas experiencing abnormally high mortalities and showed clear gross signs of OOD as defined in the formal case definition for the disease. A number of the OOD-case oysters also had direct supporting evidence from histopathology to identify tissue oedema (swelling) (as defined by Jones *et al.* 2010). However, for other OOD-cases, insufficient

tissue was available for histopathology, so their designation as OOD-cases came from their co-location with oysters exhibiting clear histopathological signs of tissue oedema. During the course of this project (2013-2016), there were only two events at different times and different locations that met the full case definition of OOD.

OOD-non-cases (controls): were oysters collected from areas experiencing no unusual mortalities, that did not fit the case definition (*i.e.* did not conform to any of the bullet pointed criteria in Box 1) and did not show signs of oedema.

OOD-suspected oysters: were collected from areas experiencing high mortality that farmers attributed to OOD. These oysters had the typical gross characteristics of mantle retraction and reduced adductor muscle function, but oedema could not be detected by histopathology. Many of these oysters were spat or juveniles. Histopathologists report that it is extremely difficult to observe the tissue oedema needed to fulfil the case definition of OOD in these life history stages.

4.1.2 Opportunistic sampling

Intensive opportunistic sampling to obtain tissue for histopathology and molecular analyses was undertaken at a number of different oyster growing sites when abnormal mortalities became apparent and were reported by farmers (Fig. 3a). These samples were collected from both adults and spat. A summary of the samples analysed in this project is shown in Table 1. Molecular approaches for pathogen discovery (next-generation sequencing – NGS) were carried out on OOD-cases and OOD-non-cases collected from different geographic locations. Findings from the NGS "discovery phase" (*Phase 1*) were then validated on a much larger sample set using a high-throughput technique – quantitative (q) PCR (*Phase 2*). The samples used for qPCR included tissue from OOD-cases and OOD-non-cases, as well as OOD-suspected oysters. They were collected from a broad range of geographic locations and at different times to negate natural differences in the normal microbiota of oysters.

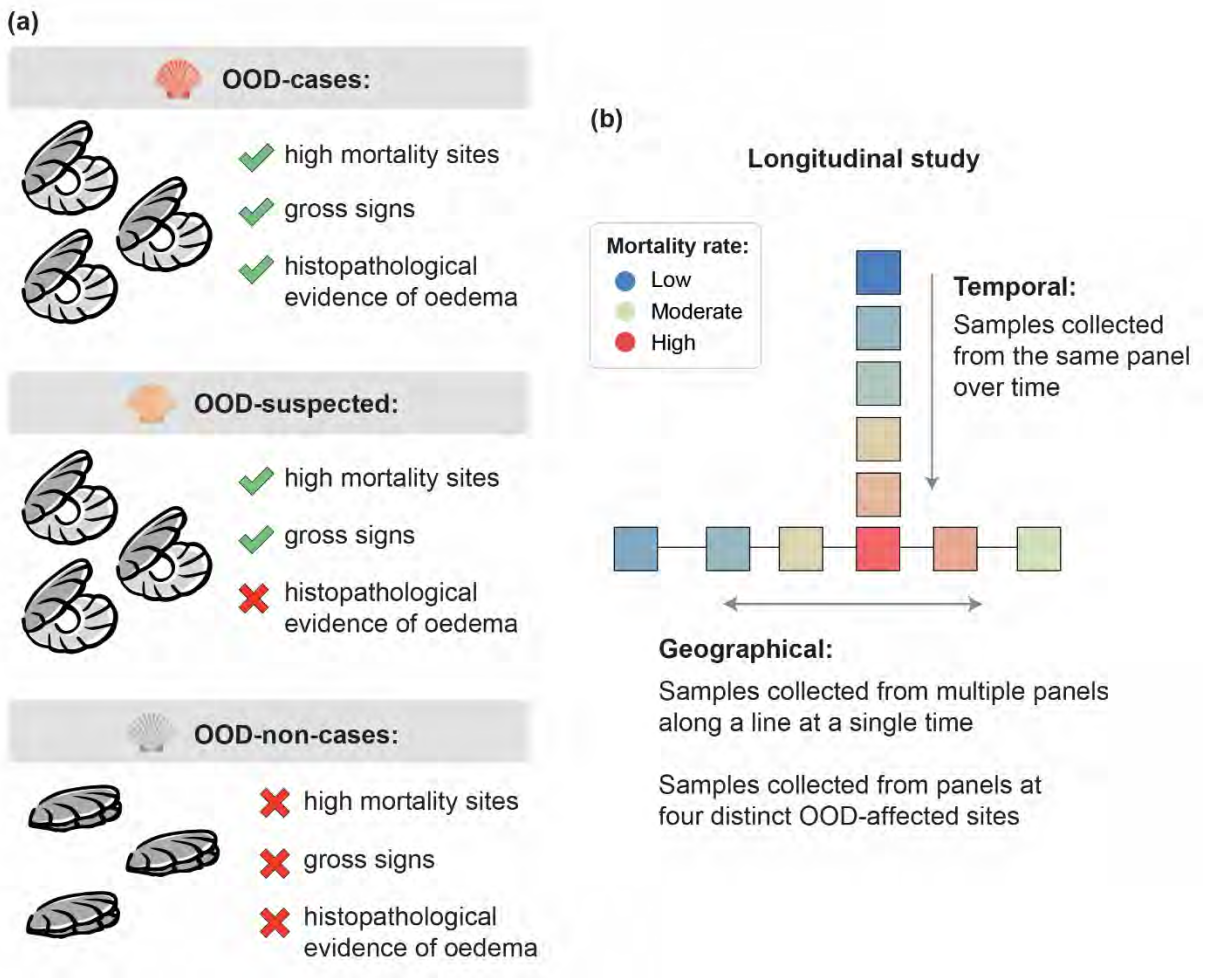

Figure 3. Sampling strategy adopted in the project. **(a)** Opportunistic sampling of oysters from areas experiencing high mortality events. **OOD-cases** were collected from areas suffering high mortalities where oysters exhibited clear gross signs of OOD (as defined in the case definition for the disease). Some OOD-cases also had histological evidence of oedema, and all come from sites were other oysters have histopathological signs of oedema. **OOD-suspected** oysters came from areas suffering high mortalities, showed gross signs of OOD but lacked histopathological confirmation of oedema. **OOD-non-cases** (non-affected control) were collected from areas of low mortality that were lacking both the gross and histopathological signs of OOD and were otherwise deemed by farmers as healthy. **(b)** Schematic diagram of the sampling strategy of the longitudinal study. Samples were collected over time and space during OOD outbreaks.

4.1.3 Longitudinal study

In addition to the opportunistic sampling conducted in OOD-affected and disease-free areas, a longitudinal sampling study was performed at four different sites within a single

embayment during a period that encompassed an entire mass mortality event. It captured a mass mortality event from before abnormal mortality became evident to the end of the event when most oysters were dead. This longitudinal study involved frequent, sequential sampling every two to four days (weather permitting) over periods of one to three months (Fig. 3b). At each site (all within 5 km of each other), samples were taken from oysters from a single line of oyster panels (*i.e.* wire cable suspended by buoys securing numerous oyster panels) (Fig. 4a). Each line held up to 80 panels of oysters along its length, with each panel separated from the next by about 1.5 to 3 m. Each panel (1 m × 1.5 m) held 45 oysters in separate mesh pockets (Fig. 4b). On each sampling day, oyster spat were collected from between 5-8 panels on each line. Whole tissue was collected for both histopathology (stored in formalin) and molecular analysis (stored in RNAlater®, Sigma-Aldrich) (Fig. 4c). Individual panels were sampled until there were insufficient oysters remaining for collection (either through mortality or sampling, or both). Along with tissue samples for laboratory analyses, mortality was recorded for each panel at each time point (Fig. 3b). The criteria for this experimental design were based on observations by farmers, who believe that mortalities begin in one panel on a line, and then spread up and down the line over time.

Oysters collected from this longitudinal study were not classified into any of the three categories defined in Fig. 3a (*i.e.* OOD-cases, OOD-suspected and OOD-non-cases). The histopathological signs of OOD are not detectable in spat even though those spat may be suffering from OOD (Fran Stephens, Fisheries WA, personal communication). Hence, we were not able to assess the oysters used in the longitudinal study in the context of the formal case definition, and histopathological analysis performed on a small subset of the samples (30 out of 659 samples) confirmed that tissue samples from spat were not suitable for the identification of oedema. However, the accompanying mortality data for the specific panels from which oysters were collected enabled the analysis of samples from the longitudinal study in terms of nucleotide sequence (contig) abundance vs. cumulative mortality.

We selected a subset of the 659 samples from the longitudinal study for examination. Sample selection was based on the quality of the stored tissue, as well as the completeness and duration of mortality data for the different panels. We focused the analysis on: (1) oysters collected from a single panel over time, where mortality rates progressively increased in that panel over time; (2) oysters collected from adjacent panels on a single line at a single time point where mortality was high in a central panel on the line and then tapered away further up and down the line; and (3) oysters from lines that experienced extremely low rates of mortality over most of the sampling period to act as a contrast to panels that experienced extremely high levels of mortality (Fig. 3b).

Figure 4. Longitudinal sampling study performed in four sites within the same embayment. At each site, samples were taken from oysters from panels from a single line **(a)**. Panels **(b)** were sampled every two to four days over periods of one to three months. **(c)** One of the sampling stations built for the collection of whole tissue for both histopathology (stored in formalin; larger tubes in the image) and molecular analysis (stored in RNAlater®; smaller tubes in the image).

Table 1. Summary of the samples analysed in the project.

Oyster condition	Location ID[a,b]	Sampling year	Life stage	High mortality	Gross signs	Histopathological evidence of oedema	Number of samples	Approach
OOD-cases	1	2006[c]	Adults	Yes	Yes	Yes	1 pool of 5	First round of NGS
OOD-cases	1	2006[c]	Adults	Yes	Yes	Yes	50	Electron microscopy
OOD-cases	2	2013	Adults	Yes	Yes	Yes	4 + 1 pool of 10	Second round of NGS
OOD-cases	2	2013	Adults	Yes	Yes	Yes	9 + 1 pool of 10	qPCR analysis
OOD-cases	4	2014	Spat	Yes	Yes	Yes	6	qPCR analysis
OOD-non-cases	3	2013	Adults	No	No	No	4 + 1 pool of 10	Second round of NGS
OOD-non-cases	3	2013	Adults	No	No	No	10	qPCR analysis
OOD-non-cases	3	2015	Adults	No	No	No	5	qPCR analysis
OOD-non-cases	4	2016	Spat	No	No	No	8	qPCR analysis
OOD-suspected	6	2013	Spat	Yes	Yes	No	5	qPCR analysis
OOD-suspected	3	2015	Adults	Yes	Yes	No	5	qPCR analysis
LS[d]	5a	2013	Spat	Yes	n/t[e]	No	134	qPCR analysis
LS[d]	5b	2013	Spat	Yes	n/t[e]	No	31	qPCR analysis
LS[d]	5c	2013	Spat	Yes	n/t[e]	No	8	qPCR analysis
LS[d]	5d	2013	Spat	Yes	n/t[e]	No	9	qPCR analysis

[a] Location 1: WA-embayment; Location 2: NT-offshore; Location 3: NT-offshore; Location 4: WA-offshore; Location 5: WA-embayment.
[b] Lower case letters indicate different sites within the same geographical location.
[c] Archived samples provided by Fisheries WA; [d] Longitudinal study; [e] Not tested.

4.2 Sample preparation

4.2.1 Next-generation sequencing (NGS)

Twenty adult oysters (10 OOD-cases and 10 non-cases) collected in 2013 at two different geographical locations were selected for next-generation sequencing (NGS). Tissue samples for NGS were stored in RNAlater® (Sigma-Aldrich) at -20 °C until processing. RNA extraction was performed separately from five tissues (mantle, gill, digestive tract, gonad and foot). Total RNA was extracted from approximately 100 mg of tissue using TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. Following resuspension, RNA was treated with DNase I (Promega) and further precipitated with 0.3 M sodium acetate (pH 5.5) and isopropanol. The concentration and quality of total RNA were checked with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). RNA samples had yields > 44 ng/µl, 260/230 ratios > 1.02 and 260/280 ratios between 2.0-2.1 (Table 2). Their quality was also confirmed using an Agilent Bioanalyser (Fig. 5).

The extracted RNA from the five tissue types of each oyster was pooled in equivalent amounts. This produced a pool of RNA samples for each of the 20 oysters. Samples used for NGS were as follows:

• Samples I1, I2, I3 and I4 correspond to RNA from the four oysters that were most severely affected by oedema (OOD-cases). These oysters were collected in 2013 from an area experiencing elevated mortalities, and showed gross and histopathological signs of OOD.

• Sample IPool10 corresponds to a pool of all 10 OOD-cases collected in 2013.

• Samples C1, C2, C3 and C4 correspond to RNA from four oysters randomly selected from the OOD-non-cases collected in 2013. These oysters were collected from areas experiencing normal (baseline) mortalities, and did not show gross or histopathological signs of OOD.

• Sample CPool10 was a pool of all 10 OOD-non-cases collected in 2013.

Table 2. Summary statistics and NanoDrop concentrations for all 20 RNA samples included in the NGS analysis (OOD-cases, I; non-cases, C).

Sample ID	Library prep method	Concentration (ng/µl)	260/280	260/230
I1	Poly-A	77.61	2.05	1.11
I1	Total-RNA	65.11	2.02	1.02
I2	Poly-A	44.72	2.06	1.42
I2	Total-RNA	43.47	2.1	1.5
I3	Poly-A	66.03	2.12	1.27
I3	Total-RNA	66.55	2.03	1.3
I4	Poly-A	44.02	2.06	1.42
I4	Total-RNA	44.47	2.12	1.28
IPool10	Poly-A	61.05	2.1	1.14
IPool10	Total-RNA	82.75	2.07	1.25
C1	Poly-A	70.45	2.06	1.43
C1	Total-RNA	69.99	2.09	1.48
C2	Poly-A	79.4	2.07	1.51
C2	Total-RNA	83.6	2.09	1.47
C3	Poly-A	62.22	2.08	1.2
C3	Total-RNA	81.2	2.1	1.24
C4	Poly-A	75.92	2.1	1.79
C4	Total-RNA	70.11	2.09	1.84
CPool10	Poly-A	76.51	2.02	1.49
CPool10	Total-RNA	80.25	2.08	1.44

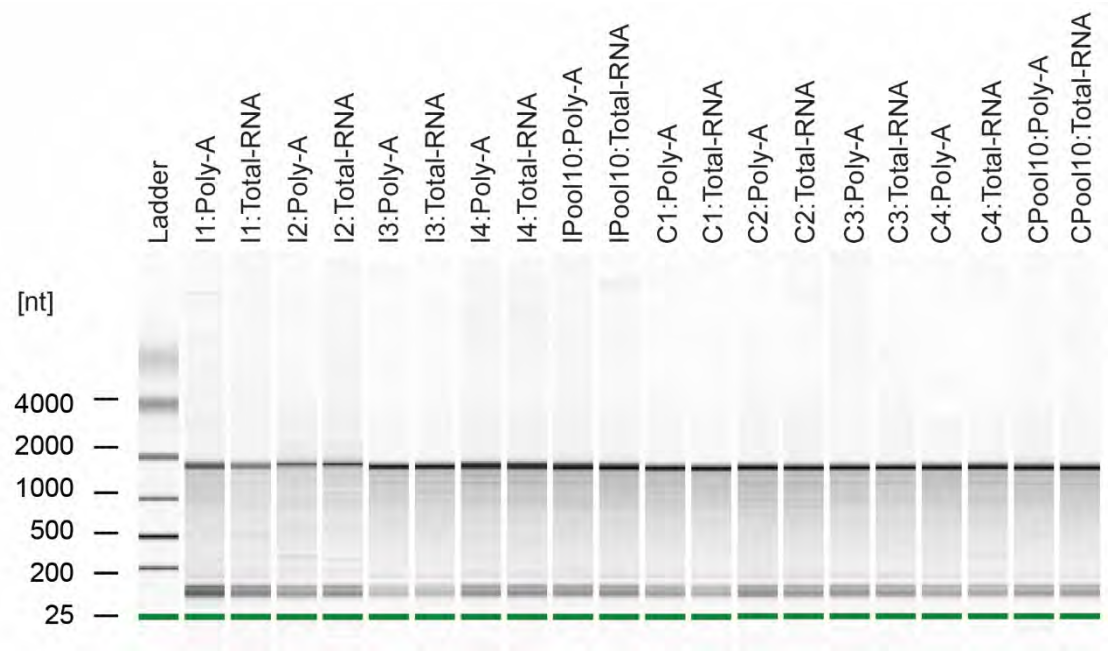

Figure 5. Bioanalyser (Aligent) gel images for the samples used to construct the 20 cDNA libraries for the second round of NGS. These images are consistent with high-quality samples for both OOD-cases (I) and non-cases (C).

4.2.2 qPCR

Forty-one samples collected opportunistically from different locations and at different life stages (spat and adults) were selected to be analysed by qPCR. They included samples from areas experiencing abnormally high mortalities with gross and histopathological signs of OOD (OOD-cases), as well as samples from areas with no abnormally high mortalities and with no gross or histopathological signs of disease (OOD-non-cases). We also included a number of samples that farmers suspected were OOD-cases, *i.e.* from areas experiencing abnormally high mortalities with gross signs of OOD, but had no histopathological evidence of tissue oedema (OOD-suspected).

A total of 16 OOD-cases, 10 OOD-suspected and 15 OOD-non-cases (control) were selected for qPCR analysis (Table 1). These samples correspond to two independent, confirmed OOD events (OOD-cases) from two distinct locations, and two suspected OOD events (OOD-suspected) from two distinct locations. The analysis also included OOD-non-cases from three different samplings at two distinct locations where oysters did not fit the formal case definition for OOD at the time of sampling. One set of OOD-cases and one set of non-cases were collected from

the same location (location 4 in Table 1) at different times. Similarly, one set of OOD-suspected (2015) and two sets of OOD-non-cases (2013 and 2015) were collected from the same location (location 3 in Table 1). The 2015 sampling in this location was performed at the same time but included different cohorts of oysters: one cohort did not experience unusual mortalities and had no gross signs of OOD (OOD-non-cases), while the second cohort experienced elevated mortalities, had gross signs of OOD but did not show histopathological evidence of oedema (OOD-suspected).

Samples for qPCR analysis were prepared following the same protocol used for NGS (section 4.2.1 above). RNA extraction of samples from adult oysters was performed separately from five tissues (mantle, gill, digestive tract, gonad and foot), while RNA extraction from spat was performed using whole-body tissue. RNA samples with yields > 185 ng/µl and 260/230 ratios > 1.2 were used for cDNA synthesis. All selected samples had 260/280 ratios between 1.9-2.3 (Appendix 1). For RNA extracted from individual tissues (adult oysters), equivalent amounts of RNA from different tissues were pooled prior to cDNA synthesis.

Complementary DNA (cDNA) was synthesized from 0.75 µg of total RNA using ImProm-II Reverse Transcription System (Promega) and 0.5 µg of random primer. Two reactions were performed per sample in a 40 µl reaction volume, according to the manufacturer's protocol. Samples were then diluted in water (5x; 200 µl final volume) and stored at -20 °C.

4.2.3 Histopathology

Samples for histopathology were dissected and fixed according to Jones *et al.* (2010). Briefly, tissue samples were fixed on-site with 10% seawater buffered formalin for 12 to 18 h. They were then drained of free fixative, packed and transported to Fisheries WA for analysis. On receipt, samples were dehydrated through a graded alcohol series, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) using standard techniques. This method has been optimised across the industry since 1994 (Humphrey *et al.* 1998; Jones 2008).

4.2.4 Transmission electron microscopy

The decision to include transmission electron microscopy (TEM) analysis of tissue from OOD-cases in this project was taken after we had collected tissue samples from OOD-case oysters. The samples from the OOD-cases that we collected were not appropriately fixed for TEM, and no further confirmed OOD-cases were reported after the decision was made to undertake TEM. Hence, we reanalysed samples from the original histopathological assessment of OOD reported in Jones *et al.* (2010). These were from the same event used to establish the case definition of OOD. Samples were processed for TEM at either Department of Fisheries in WA or the Australian Animal Health Laboratory in Geelong, Victoria. Plates from that original TEM were scanned and reanalysed in the current study.

4.3 Next-generation sequencing (NGS)

4.3.1 Sequencing approaches

An initial round of NGS was undertaken on total RNA and DNA extracted from frozen tissue stored by Fisheries WA since the initial reported OOD event in 2006. The tissue came from 5 OOD-case oysters (Table 1). Data from the initial round of NGS reflected significant degradation of both RNA and DNA after long-term storage. That data is not included in this report due to these issues with sample quality. However, the analysis of the sequence data from this initial round of NGS allowed the establishment and benchmarking of specialised NGS analysis workflows designed specifically to identify potential pathogens in OOD-cases. These workflows were developed in preparation for a second, more extensive round of NGS designed to provide a comprehensive identification of all microorganisms (including viruses) in OOD-cases and non-case oysters.

The second round of NGS was performed using a HiSeq2000 (Illumina) platform. Sequencing was performed by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales. The TruSeq protocol (Illumina) was followed for cDNA synthesis and library

preparation (see section 4.2.1 above). Two different types of library (sequencing approaches) were prepared:

(1) Poly-A: cDNA libraries prepared from Poly-A(+) RNA. Most ribosomal RNA sequences would been removed from these libraries, but eukaryote and most expressed virally-encoded sequences would have been retained;

(2) Total-RNA: cDNA libraries prepared from total RNA samples (not purified mRNA). These libraries would have retained bacterial ribosomal RNA and RNA virus sequences.

These two sequencing approaches were undertaken in an attempt to ensure that all varieties of potential pathogens that may be present in OOD-cases were detectable. One consideration when searching for infectious agents using sequencing data is that some pathogens (notably viruses) are not always readily detected via Poly-A(+) RNA (mRNA). The standard Poly-A library preparation for NGS utilises mRNA selection and therefore RNA sequences from some viral pathogens might not be contained in these selected sequences. Hence libraries of cDNA produced from total RNA samples (not purified mRNA) were also included in our analysis. Additional libraries derived from genomic DNA (as opposed to cDNA libraries derived from RNA) were not prepared because preliminary analysis of the first round of NGS revealed that the sequences generated from the DNA libraries did not return any additional potential pathogens that were not already detected in the sequences generated from RNA. Moreover, the vast majority of existing NGS studies to identify unknown pathogens use RNA not DNA as a source material, for reasons including the logic that any pathogen responsible for OOD would be making RNA if it is a viable and replicating organism causing disease (*e.g.* Epstein *et al.* 2010; Løvoll *et al.* 2010).

The resulting 20 cDNA libraries (10 Poly-A and 10 Total-RNA) were barcoded and sequenced on two separate lanes of the Illumina HiSeq2000 sequencing platform. Data produced from each of the two sequencing approaches were analysed separately using the same workflows.

4.3.2 Bioinformatic analysis

Analysis of NGS data consisted of four major steps of bioinformatic process (Fig. 6). These were:

(i) sequence quality control (QC);

(ii) *de novo* assembly;

(iii) differential abundance analysis;

(iv) annotation of differentially abundant contigs through sequence searches.

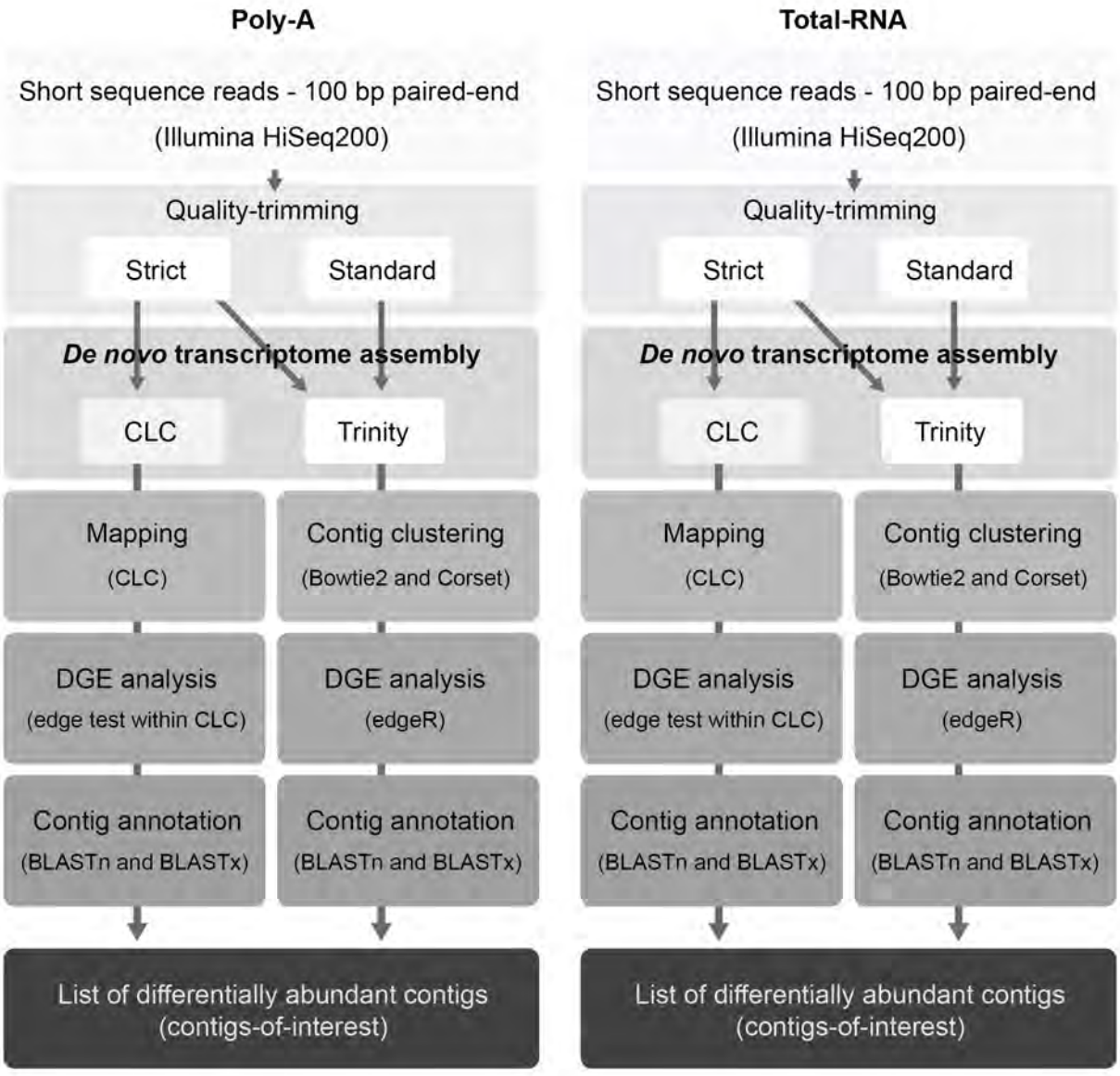

Figure 6. Overview of the bioinformatic process, including sequence quality control, assembly, annotation and differential abundance analysis.

Sequence quality control (QC)

Raw paired-end reads of 100 bp from both Poly-A and Total-RNA sequencing approaches were quality-filtered using Trimmomatic (version 0.32) (Bolger *et al.* 2014). The purpose of sequence quality control was to filter the raw sequence reads obtained from the service provider to ensure only high quality reads were retained for subsequent bioinformatic analysis. Reads were trimmed using two different sets of quality control parameters: **standard** and **strict** parameters. Sequence quality control using standard parameters trimmed reads if the average quality within a window of 4 bp was below 25. Unpaired reads and reads smaller than 30 bp were discarded. Strict quality control consisted of: a sliding window of 4 bp with an average quality score of 15, a leading quality score trimming of 28, a trailing quality score trimming of 28 and a minimum read length of 50 bp. Additional QC analysis was also conducted on reads from the strict trimming to remove low complexity sequences (homopolymer of > 10 bp) and any remaining low quality reads (or sections of reads) with a quality score threshold of 0.01 (CLC Genomics Workbench; version 7.6). The resulting quality of the trimmed reads was visualised using FastQC (version 0.10.1). Basic statistics of processed sequencing reads obtained from strict and standard trimming parameters are shown in Table 3.

Table 3. Sequencing read statistics. Basic statistics of paired-end 100 bp reads for OOD-cases (I = infected) and non-cases (C = control) oysters (n = 5 per condition). RNA reads were obtained from the oysters by using two different sequencing approaches: Poly-A or Total-RNA (grey background). Quality trimming was performed using strict (1) and standard (2) parameters.

Condition	Sequencing approach	Number of reads pre-trimming	Phred-score pre-trimming[a]	Number of reads post-trimming	Phred-score post-trimming[a]
C1	Poly-A	41,858,656	33.46	(1) 35,470,580 (2) 36,406,020	(1) 35.88 (2) 35.29
C2	Poly-A	38,604,558	33.52	(1) 32,836,656 (2) 32,104,630	(1) 35.88 (2) 35.29
C3	Poly-A	38,255,468	33.54	(1) 32,518,932 (2) 32,393,140	(1) 35.92 (2) 35.33
C4	Poly-A	43,322,406	33.43	(1) 36,697,506 (2) 37,151,116	(1) 35.85 (2) 35.25
C10Pool	Poly-A	40,976,500	33.52	(1) 34,775,574 (2) 35,027,662	(1) 35.91 (2) 35.32
I1	Poly-A	63,400,158	33.53	(1) 53,939,284 (2) 56,358,922	(1) 35.89 (2) 35.29
I2	Poly-A	49,173,008	33.49	(1) 41,702,898 (2) 43,662,010	(1) 35.88 (2) 35.29
I3	Poly-A	64,788,510	33.47	(1) 54,909,602 (2) 57,423,636	(1) 35.88 (2) 35.29
I4	Poly-A	45,479,116	33.55	(1) 38,907,048 (2) 40,045,538	(1) 35.86 (2) 35.27
I10Pool	Poly-A	67,336,386	33.46	(1) 57,178,970 (2) 58,113,710	(1) 35.84 (2) 35.25

Table 3 continued

Condition	Sequencing approach	Number of reads pre-trimming	Phred-score pre-trimming[a]	Number of reads post-trimming	Phred-score post-trimming[a]
C1	Total-RNA	46,214,174	35.84	(1) 42,929,254 (2) 42,165,824	(1) 37.30 (2) 36.69
C2	Total-RNA	37,308,218	35.82	(1) 34,593,260 (2) 32,277,548	(1) 37.33 (2) 36.73
C3	Total-RNA	50,203,254	35.60	(1) 46,267,228 (2) 43,728,726	(1) 37.20 (2) 36.59
C4	Total-RNA	48,483,388	35.72	(1) 44,763,970 (2) 41,954,952	(1) 37.28 (2) 36.67
C10Pool	Total-RNA	39,104,336	35.54	(1) 35,900,760 (2) 34,496,590	(1) 37.22 (2) 36.61
I1	Total-RNA	43,907,768	35.90	(1) 40,976,948 (2) 41,621,608	(1) 37.29 (2) 36.68
I2	Total-RNA	44,726,920	35.76	(1) 41,431,370 (2) 42,241,540	(1) 37.26 (2) 36.65
I3	Total-RNA	42,041,754	35.77	(1) 38,991,630 (2) 39,470,932	(1) 37.25 (2) 36.64
I4	Total-RNA	45,956,854	35.65	(1) 42,434,532 (2) 42,437,970	(1) 37.19 (2) 36.57
I10Pool	Total-RNA	46,609,054	35.74	(1) 43,156,980 (2) 42,110,224	(1) 37.24 (2) 36.63

[a] Mean quality score for R1 and R2 reads.

De novo *assembly*

The second stage of bioinformatic analysis was to assemble the processed RNA reads generated by strict and standard trimming into longer stretches of nucleotides ("contiguous sequences" or "contigs"). During *de novo* assembly, raw sequences are merged into contigs based on mutual sequence overlap. Thus, issues caused by lack of genomic information for non-model species, which is the case of *P. maxima*, are circumvented because a reference genome is not required.

Processed RNA reads generated by strict and standard trimming were independently assembled into reference scaffolds using CLC Genomics Workbench (version 7.6) and Trinity (version trinityrnaseq_r20140717). CLC assemblies were conducted using RNA reads generated by strict parameters only. Assembly parameters consisted of optimal word and bubble sizes determined by CLC assembly algorithms (25 and 50, respectively) and a minimum contig size of 400 bp. This produced two *de novo* assemblies, one for each of the sequencing approaches undertaken:

(1) CLC-Poly-A-strict: reads from the Poly-A sequencing approach, filtered by strict trimming parameters;

(2) CLC-Total-RNA-strict: reads from the Total-RNA sequencing approach, filtered by strict trimming parameters.

Trinity assemblies were performed using reads produced by both strict and standard trimming parameters (standard assembly parameters, minimum contig length = 400 bp). Four assemblies were generated, two for each of the sequencing approaches undertaken:

(3) Trinity-Poly-A-strict: reads from the Poly-A sequencing approach, filtered by strict trimming parameters;

(4) Trinity-Poly-A-standard: reads from the Poly-A sequencing approach, filtered by standard trimming parameters;

(5) Trinity-Total-RNA-strict: reads from the Total-RNA sequencing approach, filtered by strict trimming parameters;

(6) Trinity-Total-RNA-standard: reads from the Total-RNA sequencing approach, filtered by standard trimming parameters.

The quality of the six assemblies was evaluated according to basic statistics (khmer tools; https://github.com/dib-lab/khmer/tree/master/sandbox) and the correspondence of contigs with original sequencing reads (Bowtie 2, default parameters) (Langmead & Salzberg 2012). N50 value, mean length, maximum length and number of contigs, as well as the overall alignment rate were compared among the different assemblies.

Differential abundance analysis

To maximise the discovery of sequences associated with OOD, the six assemblies generated by CLC or Trinity (2 from CLC and 4 from Trinity) were used for differential abundance analysis. Assemblies were analysed independently following assembler-specific pipelines. For assemblies generated by CLC (CLC-Poly-A-strict and CLC-Total-RNA-strict), the raw reads of each library were mapped back to their respective scaffolds using CLC Genomic Workbench Legacy read mapping algorithms (maximum number of mismatches of 2, a minimum length fraction of 0.8, a minimum similarity fraction of 0.8 and a maximum number of hits for a read of 20). Read counts were calculated as RPKM (Reads Per Kilobase per Million mapped). This provided an estimation of the abundance of sequence reads for each contig over all libraries, allowing us to calculate the relative abundance of potential pathogen sequences.

Assembled contigs generated by Trinity were combined into clusters (for each individual assembly: Trinity-Poly-A-strict, Trinity-Poly-A-standard, Trinity-Total-RNA-strict and Trinity-Total-RNA-standard) in order to reduce contig redundancy and facilitate downstream analysis (Corset, version 1.03) (Davidson & Oshlack 2014). To do this, processed read pairs from each

library were individually mapped back to their respective Trinity assembly (Bowtie 2, strict mapping parameters). For example, the subset of Poly-A reads generated by strict trimming parameters was mapped back to the Trinity-Poly-A-strict assembly, while the subset of Total-RNA reads produced by standard trimming parameters was mapped back to the Trinity-Total-RNA-standard assembly. Assembled contigs were then clustered based on the proportion of shared reads and abundance patterns for each assembly. The cluster-level count data generated by Corset were processed using the edgeR Bioconductor package, testing for differences in contig abundance between the OOD-case and OOD-non-case (control) oysters that were used for NGS. Clusters with no detectable reads in more than 40% of the analysed samples were discarded from statistical analyses (that is, detection was required in at least 3 out of the 5 OOD-case samples sequenced). Contigs were considered to be differentially abundant (OOD-cases vs. OOD-non-cases) at $p < 0.05$ with FDR correction (FDR < 0.05).

Annotation of differentially abundant contigs

The list of differentially abundant contigs (contigs-of-interest) from each of the six assemblies (2 from CLC and 4 from Trinity) was subjected to thorough searches against public domain sequence databases to identify matching species and assign tentative gene IDs. Contigs-of-interest derived from CLC assemblies (CLC-Poly-A-strict and CLC-Total-RNA-strict) were searched against custom databases including over 4 million sequences of viruses, bacteria and other microbes, as well as molluscan sequences. These databases encompassed PATRIC BRC, VIPR BRC Protein, Braembl Uniprot Swiss-prot, PATRIC BRC Protein Transcriptomics, NCBI Mollusca Non-redundant, NCBI Viridae Protein, PHI-base Protein, Fungal DFVF All FVF, NCBI Mollusca Non-redundant nt, VIPR BRC All Nucleotide, NCBI Viridae Genomic and NCBI 16S Microbial.

Contigs-of-interest derived from Trinity assemblies (Trinity-Poly-A-strict, Trinity-Poly-A-standard, Trinity-Total-RNA-strict and Trinity-Total-RNA-standard) were annotated using NCBI

nucleotide (nt; BLASTn) and non-redundant (nr; BLASTx) databases (complete NCBI databases including all nucleotide and protein entries). Sequence similarity searches were performed with Blast2GO PRO (version 3.1.3), using an e-value cut-off of 10^{-10}.

Once similarity searches were performed, results were compiled and the most significant BLASTn and BLASTx hits (based on lowest e-value and highest bit score) to each contig for each scaffold was retained. Contigs with matches to known molluscan or other animal sequences were discarded. Only contigs found at higher abundance in OOD-cases (relative to OOD-non-cases) were considered for downstream analysis. In addition, contigs were selected for further analysis if high abundance (RPKM) was observed in at least 3 out of the 5 OOD-case samples analysed by NGS.

The majority of the contigs-of-interest were classified as unknown or unidentified since BLASTn and BLASTx searches did not return any robust homologous sequences. We cannot determine whether these contigs represent potential, uncharacterised pathogen sequences or uncharacterised oyster gene sequences because the complete genome of *P. maxima* is unavailable. Given that the major aim of this project is to develop a diagnostic test to determine the presence of OOD, we cannot rule out the possibility that these unknown/unidentified sequences are associated with OOD and so could be useful in diagnostics. Therefore, unannotated contigs were retained for downstream analysis when they were found at much higher abundance in OOD-cases to OOD-non-cases (fold-change ≥ 30) (Fig. 7).

To reduce contig redundancy across the different sets of analyses (2 assemblies generated by CLC and 4 produced by Trinity), each list of contigs-of-interest was searched against the other assemblies using BLASTx (version 2.2.28+; e-value cut-off 10^{-10}). For example, the list of contigs-of-interest resulting from the Trinity-Poly-A-standard analysis was searched against the other two Poly-A assemblies (Trinity Poly-A-strict and CLC-Poly-A-strict). The same procedure was conducted for each of the lists of contigs-of-interest produced from Total-RNA assemblies. Only unique new contigs were considered for downstream qPCR analysis (Fig. 7).

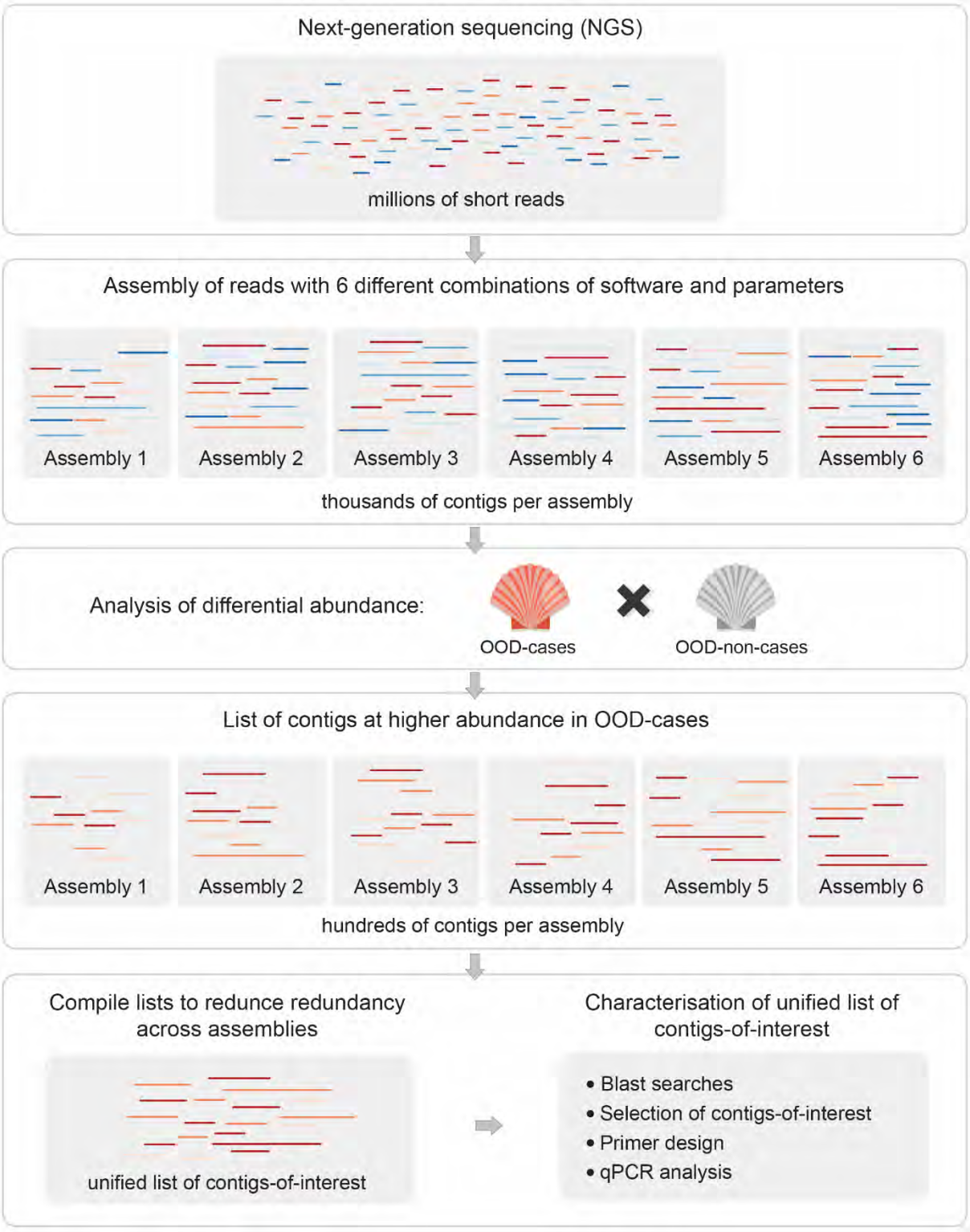

Figure 7. Selection of the list of contigs-of-interest from six independent assemblies generated by CLC Genomics Workbench or Trinity. The diagram shows the steps undertaken for each of the assemblies and the production of the unified list of nucleotide sequences (contigs-of-interest) that was further analysed by qPCR.

4.4 qPCR analysis

4.4.1 Selection of contigs-of-interest from NGS data

qPCR analysis was performed to verify the results of NGS by investigating a far larger sample set including OOD-cases and controls (OOD-non-cases) from different locations and at different life history stages (spat and adults) (Table 1). The sample set also included a number of OOD-suspected oysters.

Nucleotide sequences evaluated by qPCR were selected based on a list of "contigs-of-interest" generated by the independent analysis of the six NGS assemblies (2 CLC assemblies and 4 Trinity assemblies), according to the following criteria:

(i) Contig was found at significantly higher abundance (RPKM) in OOD-cases relative to OOD-non-cases;

(ii) High abundance must be observed in at least 3 out of the 5 OOD-case samples analysed by NGS for any given contig;

(iii) Contig must not match known molluscan or other animal sequences;

(iv) Contig must match microbial sequences and have a fold-change ≥ 2 (OOD-cases vs OOD-non-cases), or, Contig does not match any sequences in the available databases, but has a fold-change ≥ 30 (OOD-cases vs. OOD-non-cases);

(v) Contig sequence enables the design of high-quality primers for qPCR analysis.

4.4.2 Primer design

qPCR primers for the selected contigs-of-interest were designed using Primer3 software. A total of 134 primer pairs for contigs-of-interest and 7 for potential reference genes were designed. Primers for potential reference genes were designed based on sequences for *P. maxima* available at NCBI. They were sequences commonly used as reference genes, including elongation factor 1α

and ribosomal proteins. The stability of these genes was tested by qPCR prior to relative abundance analysis.

4.4.3 Preliminary test of primer pairs

A preliminary test was undertaken to identify primer pairs that did not amplify at the desired target annealing temperature for qPCR (60 °C) and those that produced non-specific amplification products or secondary structures (*e.g.* primer dimers). In addition to testing primer pairs for the selected contigs-of-interest, this initial analysis also screened for two viral pathogens that affect molluscs in Australia: Ostreid Herpes Virus (OsHV-1) and Abalone Herpes virus. Primer sequences for these two pathogens were sourced from the literature (3 for Oyster Herpes virus and 1 for Abalone Herpes virus) (Corbeil *et al.* 2010; Pepin *et al.* 2008). The design of the primer pair for Abalone Herpes virus was based on a different assay type (TaqMan®) that requires forward and reverse primers and a probe (Corbeil *et al.* 2010). Hence, forward and reverse primers for Abalone Herpes virus were tested in-silico using Beacon Designer and IDT OligoAnalyzer 3.1 in order to assess their suitability for SYBR® Green assays. The evaluation of amplicon size, length of the primers, annealing temperature, GC (%) content and production of secondary structures (*e.g.* primer self-dimers, cross-dimers and hairpins) indicated that this primer pair could also be used in SYBR® Green assays.

qPCR assays were conducted in duplicate on a Bio-Rad CFX Real-time system (Bio-Rad). The 10 µl reaction mixtures contained 5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM of each primer (forward and reverse), 3.4 µl PCR grade water and 1 µl master cDNA (pool of cDNA from multiple oysters; diluted 1:9). The cycling program used consisted of 3 min at 95 °C followed by 40 cycles of 95 °C for 10 s, 30 s at 58 or 60 °C and 30 s at 72 °C. Melting curve analysis was performed at the end of the qPCR cycles to confirm primer specificity by collecting fluorescence data between 65 - 95 °C at 0.5 °C increments. Amplification data were analysed using the Bio-Rad CFX Real-time system software to obtain Cq values.

Of the 134 primer pairs for contigs-of-interest, 116 produced a single amplification product and exhibited average Cq values between 17 and 33. Six out of the seven primer pairs designed for potential reference genes also produced a single amplification product. None of the samples from OOD-cases exhibited any amplification for either OsHV-1 or Abalone Herpes virus. Contigs and primer sequences analysed by qPCR are listed in Appendix 2.

4.4.4 Primer optimisation

The assessment of reaction efficiency for each of the primer pairs was performed on a LightCycler® 480 II (Roche). Standard curves were generated in triplicate for each primer pair using five four-fold serial dilutions of a master cDNA sample as template (pool of cDNA from multiple oysters). Three microliter qPCR reactions were prepared in triplicate in 384-well plates using an epMotion® 5070 automated liquid handling robot (Eppendorf) and an Echo® 550 Liquid Handler (Labcyte). Each reaction mixture contained 1.5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM each primer (forward and reverse), 0.8 µl molecular grade water and 0.5 µl master cDNA (diluted 1x, 4x, 16x, 64x and 256x). The cycling program consisted of 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 6 s. Melting curve analysis was performed at the end of the qPCR cycles to confirm primer specificity by collecting fluorescence data between 65 - 95 °C at 0.5 °C increments.

Amplification data were analysed using the LightCycler® 480 Real-Time PCR System (version 1.5.1.62) to obtain Cq values and calculate the reaction efficiency of each primer pair. Out of the 116 primer pairs tested, 92 (86 primers for contigs-of-interest and 6 for potential reference genes) exhibited reaction efficiencies (E) between 90% and 110% and were used for qPCR analysis. The list of high-quality primer pairs and their reaction efficiency values is shown in Appendix 2.

4.4.5 qPCR assays

qPCR assays were conducted on the 92 primer pairs described above against 41 cDNA samples from OOD-cases, OOD-suspected oysters and OOD-non-cases (non-affected controls). Three microliter qPCR reactions were prepared in duplicate in 384-well plates using an epMotion® 5070 automated liquid handling robot (Eppendorf) and an Echo® 550 Liquid Handler (Labcyte). Each reaction mixture contained 1.5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM each primer, 0.8 µl molecular grade water and 0.5 µl cDNA template (diluted 5x). qPCR assays were performed on a LightCycler® 480 II (Roche) using the same cycling conditions as used for primer optimisation: 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 6 s. Melting curve analysis was performed at the end of the qPCR cycles (65 - 95 °C at 0.5 °C increments). qPCR amplification data were analysed using the LightCycler® 480 Real-Time PCR System (version 1.5.1.62) to obtain Cq values.

Reference gene stability was assessed using the web-based RefFinder platform, which integrates results from different tools (Xie *et al.* 2012). We tested the abundance levels of six potential reference genes (Appendix 3). The geometric means of elongation factor 1α (EF1α), ribosomal protein L8 (RPL8) and ribosomal protein L17 (RPL17) were found to be the most stable combination and so these genes were used as references (geNorm stability value = 0.081; NormFinder stability value = 0.023; Stability value by ΔCt method / Average SD = 0.26) (Appendix 3). Given that there is conjecture about the validity of using host reference genes to normalise abundance data for potential pathogens infecting those hosts, we have analysed qPCR data as both Cq values (as an estimative of absolute abundance) and relative abundance, by normalising changes in abundance against the geometric mean of the Cq values of the three reference genes (Livak & Schmittgen 2001; Schmittgen & Livak 2008).

4.5 Transmission electron microscopy (TEM)

In late 2006, as part of the initial investigation into the OOD outbreak, glutaraldehyde-fixed tissue samples of affected pearl oysters were prepared by the Department of Fisheries in WA (Department of Agriculture and Food, Western Australia - DAFWA), embedded in araldite and examined by Transmission Electron Microscopy (TEM). Examination was initially undertaken by Dr. Brian Jones at Murdoch University. There are no records or micrographs surviving from the examination at Murdoch University.

Examination continued at DAFWA and samples were also sent to Alex Hyatt at Australian Animal Health Laboratory, Geelong. Alex Hyatt was then brought to Perth for a face-to-face discussion and examination of all of the available micrographs from a range of pearl oyster tissues from the OOD-affected areas. No conclusions were reached at that time.

As the investigation progressed, and specifically when Chlamydia-like sequences were obtained in 2008 (Crockford & Jones 2012), further TEM was attempted and Chlamydia-like bodies were found. This finding was reported (without further elaboration) by Crockford & Jones (2012) in support of evidence for the presence of two Chlamydiales detected by molecular methods in OOD-affected oysters.

In 2015, when the AAH subprogram requested further TEM work to be undertaken, locating the already fixed and embedded material was the first priority but ultimately proved unsuccessful - communication with Fisheries WA was intermittent because staff had other priorities around moving the Fish Health Unit of the Department of Fisheries from DAFWA to a new site and Dr. Michael Snow (FRDC coinvestigator) took six months leave. In addition, it transpired that records at DAFWA and the Fish Health Unit were incomplete. The uncut blocks and electron micrographs left by Dr. Jones and Dr. Crockford could not be found. To compound the problem, the original electron microscope notebooks held by the DAFWA Electron Microscope Unit could not be found. These books contained all user records, dates and codes used in blocks as

well as details of each micrograph exposure. This meant that the extant film quarter-plate negatives (which still exist) could not be linked back to original case numbers or blocks.

Consequently, Dr. Jones located his own laboratory diaries in which the details of his TEM examinations including negative numbers and magnifications were recorded. This gave a time frame for negatives of OOD-cases, which included those taken by Dr. Jones and Dr. Crockford. From these notes, a series of 50 negatives were identified as being of OOD-cases. These quarter-plate negatives were commercially scanned and provided to Dr. Jones in digital form. The fresh set of analysis was performed on these materials retrieved from the original 2006 preparation.

5. RESULTS AND DISCUSSION

5.1 Next-generation sequencing (NGS)

Non-technical summary: We used next-generation nucleotide sequencing (NGS) to sequence the genetic material (RNA) from 10 OOD-cases and 10 non-cases. Raw NGS data come as millions of short strings of nucleotides called "reads" (combinations of A's, G's, T's and C's up to about 100 nucleotides long). That data (almost one billion reads) were processed to produce much longer continuous strings of nucleotides ("contigs") that could be compared between OOD-cases and non-cases (non-affected controls). Six different processes to assemble the reads into longer contigs were developed using different combinations of software and parameters. These assemblies included 27,000 to 76,000 contigs each, with lengths ranging from 400 to 28,000 nucleotides long. By counting the number of copies of each contig in assemblies from OOD-cases and non-cases we identified a set of contigs (referred to here as contigs-of-interest) that were more abundant in the OOD-cases, and so might be potentially associated with the presence of OOD.

5.1.1 Sequence Assemblies

We produced 20 cDNA libraries from OOD-cases and OOD-non-cases. These libraries were sequenced and the resulting reads assembled into contigs so that we could compare the abundance of each contig in OOD-cases vs. non-cases. Poly-A library sequencing yielded 493 million paired-end reads (100 bp), while the sequencing of Total-RNA libraries resulted in 445 million paired-end reads (100 bp). Standard quality trimming retained 87% (429 million; average phred score = 35.29) of the reads from the Poly-A sequencing and 91% (403 million; average phred score = 36.65) from the Total-RNA sequencing. Strict quality trimming retained 85% (419 million; average phred score = 35.88) of the dataset generated by Poly-A sequencing and 93% (411 million; average phred score = 37.26) of the one produced by Total-RNA sequencing (Table 3).

The filtered high-quality sequencing reads were assembled into contigs using two different software packages: CLC Genomics Workbench and Trinity. We were able to maximise the chances of finding OOD-associated sequences in our analysis by using these two assemblers and testing the effects of different parameters (standard vs. strict trimming). We generated a total of six assemblies

(scaffolds), three for each sequencing approach (Poly-A and Total-RNA). Table 4 summarises the basic statistics for each of the six assemblies, as well as the correspondence of contigs with original sequencing reads.

Trinity was able to produce assemblies with longer contigs compared to the ones generated by CLC Genomics Workbench. Contigs assembled by Trinity had slightly higher N50 values, as well as higher mean and maximum lengths (Table 4). This was observed for both sequencing approaches (Poly-A and Total-RNA). The most substantial differences between the two sets of assemblies (Trinity vs. CLC Genomics Workbench) were in terms of the correspondence of short sequencing reads with the assembled contigs, including total number of bases that were assembled and the overall alignment rate. Assemblies produced by Trinity comprised approximately twice as many bases than the ones generated by CLC Genomics Workbench (Table 4). In addition to the best assembly metrics, Trinity assemblies had the highest alignment rates to the processed reads. Trinity Total-RNA assemblies showed overall alignment rates of 87% (strict trimming) and 98% (standard trimming) compared to the 60% alignment rate observed for the CLC Total-RNA assembly (strict trimming). Such differences in alignment rates were even more pronounced for the Poly-A assemblies. While CLC Poly-A assembly (strict trimming) showed 21% alignment rate with the original short-read sequences, Trinity Poly-A assemblies had alignment rates more than four times higher – 89% (strict trimming) and 92% (standard trimming) (Table 4). These findings indicate a strong representation of the original short-read sequences in the contigs assembled by Trinity.

Table 4. Assembly statistics. Assessment of the assemblies for *Pinctada maxima* generated from different subsets of processed reads and by different software packages (minimum contig length of 400 bp).

	CLC-Poly-A-strict	Trinity-Poly-A-strict	Trinity-Poly-A-standard	CLC-Total-RNA-strict	Trinity-Total-RNA-strict	Trinity-Total-RNA-standard
Assembly details						
Sequencing approach	Poly-A	Poly-A	Poly-A	Total-RNA	Total-RNA	Total-RNA
Processed reads	Strict	Strict	Standard	Strict	Strict	Standard
Assembly software	CLC Genomics Workbench	Trinity	Trinity	CLC Genomics Workbench	Trinity	Trinity
Statistics						
N50 (bp)	912	1,122	1,203	1,326	1,376	1,338
Mean length (bp)	867	962	999	1,058	1,093	1,069
Median length (bp)	638	642	653	756	812	781
Maximum length (bp)	22,998	27,423	28,871	16,436	20,233	20,324
Number of contigs	27,065	46,161	55,649	43,295	76,049	76,107
Total assembled bases (bp)	23,474,287	44,383,634	55,624,779	45,832,730	83,155,381	81,379,867
Alignment rate	21%	89%	92%	60%	87%	98%

Trinity clearly yielded the best assembly metrics. However, for completeness, we still considered the assemblies produced by CLC Genomics Workbench for differential abundance analysis. The six assemblies were independently used to compare contig abundance between OOD-cases and non-cases. Contigs assembled by Trinity were first grouped into gene clusters and cluster-level count data were then processed using edgeR. Gene clusters with no detectable abundance in more than 40% of the OOD-case samples were discarded. Abundance analyses of the assemblies produced by CLC Genomics Workbench were performed using CLC built-in functions.

5.1.2 Relative abundance analysis

The abundance of sequences in OOD-cases and OOD-non-cases (non-affected controls) was compared in order to identify contigs that were more prevalent in OOD-cases. This comparison was performed for each of the six assemblies generated (Fig. 7). Figure 8 shows the results of the differential abundance analyses using two assemblies generated by Trinity: Trinity-Poly-A-standard and Trinity-Total-RNA-standard. Analyses of abundance levels for the 3,482 gene clusters produced by Poly-A sequencing (Fig. 8a) and the 25,172 generated by Total-RNA sequencing (Fig. 8c) revealed that OOD-cases exhibited substantially different molecular profiles compared to non-affected control oysters (OOD-non-cases). As a result of these differences, NMDS plots showed a clear spatial separation between these two groups (Figs. 8a and 8c). Of 3,482 gene clusters identified by the Poly-A sequencing, 233 were found to be differentially abundant between OOD-cases and non-cases (FDR-adjusted $p < 0.05$) (Fig. 8b). A total of 120 gene clusters were found at higher abundance in OOD-cases. In terms of the Total-RNA sequencing, 2,798 gene clusters showed differential abundance between OOD-cases and non-cases, of which 1,315 had higher abundance in OOD-cases (FDR-adjusted $p < 0.05$) (Fig. 8d).

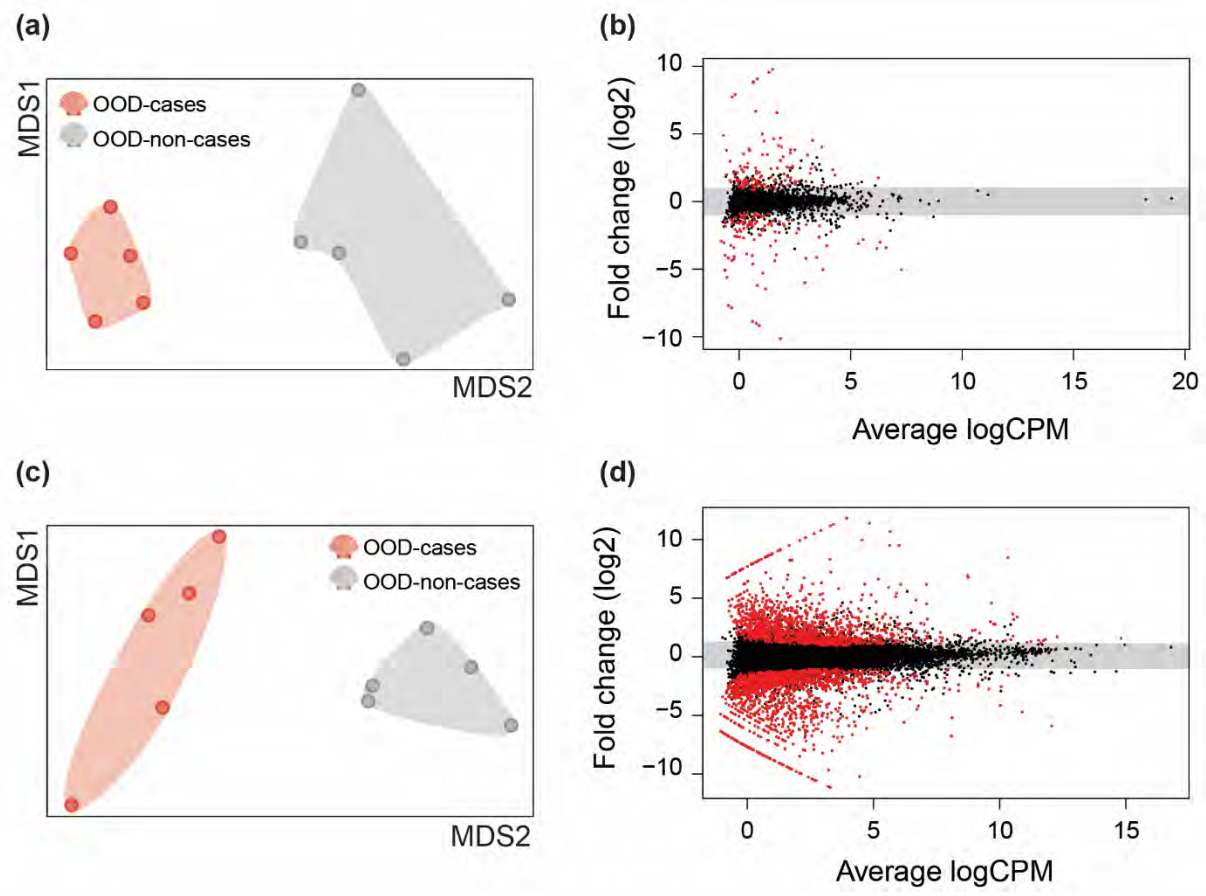

Figure 8. Molecular profiles of *Pinctada maxima*. Results from Poly-A sequencing are shown in **(a)** and **(b)**, and those from Total-RNA sequencing are presented in **(c)** and **(d)**. Multidimensional scaling (MDS) plots summarising the abundance of all contigs identified in OOD-cases (in red) and OOD-non-cases (in grey) by the Poly-A **(a)** and Total-RNA **(c)** sequencing approaches. Each data point represents an individual sample. MA plots displaying contigs identified in *P. maxima* by the Poly-A **(b)** and Total-RNA **(d)** sequencing approaches (fold change calculated as OOD-cases/non-cases). Differential abundance analysis was performed using edgeR. Contigs that were significantly differentially abundant at $p < 0.05$, FDR < 5% are displayed in red. The grey area delimits the interval where differences in abundance are not significant.

Results from the differential abundance analyses of each of the six independent assemblies were combined to reduce redundancy in the datasets. This yeilded a comphrehensive listed of nucleotide sequences (referred to here as contigs-of-interest) that were found at significantly higher levels in OOD-cases compared to non-cases (Fig. 7) (Appendix 4). These nucleotide sequences were filtered to remove duplicate entries (homologous contigs produced by more than one

assembly) and were then subjected to BLAST searches against public databases in an attempt to match these sequences with known infectious agents. These searches returned matches to potential pathogens (including matches to different strains within a species and different species within a genus), as well as a numerous unknown or unidentified sequences (no match to any sequences in the available databases). However, it is stressed that a match to a specific sequence from available sequence databases does not imply that the sequence found in pearl oysters is from exactly the same species represented in the databases. In most cases, it is likely that our sequences represent very closely related, but not identical, species to those listed in the databases.

Unknown or unidentified sequences made up the majority of the entries in our list. These sequences could not be identified as originating from known viruses, bacteria or other potential pathogens, but still appeared at far higher abundance in OOD-cases compared to non-cases (Appendix 4).

5.2 qPCR validation of contigs-of-interest

Non-technical summary: We used qPCR to more comprehensively investigate 86 contigs-of-interest that were initially identified in the small number of oysters analysed by NGS. qPCR is a high throughput method for measuring contig abundance within a sample. qPCR was used to measure the abundance of the 86 contigs-of-interest in a far larger sample set (41 oysters) comprising OOD-cases, non-cases and OOD-suspected oysters. This qPCR analysis refined the list of 86 contigs-of-interest down to 17 contigs-of-interest that were more abundant in the larger sample set of OOD-cases and OOD-suspected oysters compared to non-affected controls (OOD-non-cases). Among these 17 nucleotide sequences that qPCR tentatively associated with OOD, five were present in the majority of oysters from sites experiencing mass mortality events with clear gross and histopathological signs of OOD (OOD-cases). None of the sequences identified all OOD-cases. However, there were some combinations of two sequences that identified up to 75% of OOD-cases and none (or less than 10%) of OOD-non-cases. These combinations also identified many (up to 60%) of the oysters that were suspected of suffering from the disease but lacked histopathological confirmation of OOD (OOD-suspected).

qPCR was used to evaluate the list of contigs-of-interest identified by NGS. The initial list of contigs-of-interest represented those found at higher abundance in the small set of OOD-cases (4 individual oysters + a pool of 10 oysters) from a single geographical location. We undertook qPCR analyses to test whether the apparent association between the prevalence of these contigs-of-interest and the occurrence of OOD was also evident in a larger sample set, including 16 OOD-cases, 15 non-cases and 10 OOD-suspected oysters from numerous locations (Table 1).

qPCR data analysis was performed in the context of both absolute and relative contig abundance. There is some conjecture about the validity of using host reference genes to normalise abundance data for potential pathogens infecting those hosts. Hence, we analysed qPCR data as both raw Cq values (as an estimative of absolute abundance) and abundance relative to stable reference genes. Relative abundance of each contig-of-interest was calculated by normalising raw Cq values against the geometric mean of the Cq values of reference genes. To confirm that both methodologies provided comparable results, correspondence between absolute and relative abundance was tested by Pearson correlation analysis and linear models. These tests showed a strong concordance between the results generated by both methods of data analysis for all contigs ($p < 0.05$) (Fig. 9). Since there was no substantial difference between conclusions drawn from these two ways of displaying the data, and we primarily present data in this report as Cq values.

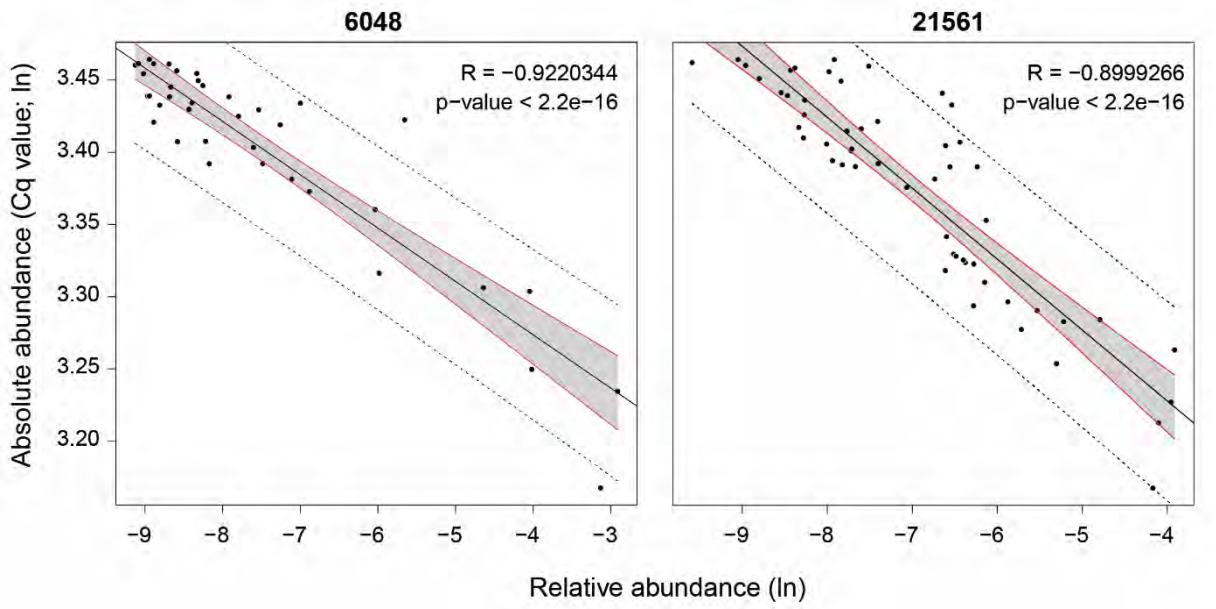

Figure 9. Comparison of contig abundance using absolute and relative quantification methods. Absolute abundance was estimated based on Cq values, while relative abundance was calculated by the normalisation of Cq values with oyster reference genes. Pearson correlation analysis was carried out on ln-transformed values (natural logarithm) for each contig-of-interest. The data show a strong correlation between the two quantitative methods. These regressions represent negative correlations because lower Cq values correspond to higher contig abundance. Solid black lines indicate linear regressions, red lines indicate confidence intervals (95%) and dotted lines indicate upper and lower limits of the data (prediction interval; 95%).

qPCR evaluation of 86 candidate contigs-of-interest against tissue samples from 41 oysters identified a set of 17 contigs-of-interest that were far more abundant in OOD-cases and OOD-suspected oysters than in non-affected controls (OOD-non-cases). Contigs-of-interest were added to this refined list when:

(i) either absolute or relative quantitative analysis showed statistically significant differences between OOD-cases/suspected oysters and non-cases (Student's t-test, $p < 0.05$);

(ii) positive amplification was observed in oysters from at least 2 out of the 4 sampling sites (2 × OOD-cases and 2 × OOD-suspected) in order to account for differences between locations;

(iii) qPCR amplification (Cq ≤ 35) could be detected in 50% of the OOD-cases/suspected oysters and in less than 10% of the OOD-non-cases (non-affected controls). Positive amplification was initially based on Cq values (absolute abundance) and was confirmed by relative abundance.

Examples of the abundance patterns identified for the 17 selected contigs-of-interest are shown in Figure 10. The abundance profiles of all 86 contigs-of-interest tested by qPCR is shown in Appendix 5. The prevalence of some of the 17 contigs-of-interest (*e.g.* 27030 and 43053) was exclusive to OOD-cases and OOD-suspected oysters. That is, they were not amplified in non-affected control oysters (OOD-non-cases) (Fig. 10a). In other cases, one or two samples from OOD-non-cases also showed amplification but the contig was amplified in many more OOD-cases and OOD-suspected oysters (Fig. 10b).

All of the 17 contigs-of-interest that were strongly associated with OOD were initially categorised as unknown or unidentified. BLASTn and BLASTx searches of the contig sequences using high stringency search parameters did not return any robust homologous matches to any known sequences in publically available sequence databases (complete NCBI nt and nr databases). Further BLASTn and BLASTx searches using far less stringent parameters were able to match some of the sequences to known genes or species (Table 5). The majority of the matches were to oyster or other animal sequences (*e.g.* sequences from amphipods and fish). Two contigs-of-interest showed some homology with bacterial and fungal sequences. Contig 616 appeared to incorporate a conserved BAR-domain superfamily domain and was homologous to a haloacid dehalogenase from the marine bacteria *Neptuniibacter caesariensis*. Contig 27036 had some homology with a carboxylic ester hydrolase from the hemibiotrophic fungus *Colletotrichum higginsianum*. However, given that all of these matches used low stringency searches, these identifications have a low degree of confidence. The complete BLAST results for each of the 17 contigs-of-interest are shown in Appendix 6.

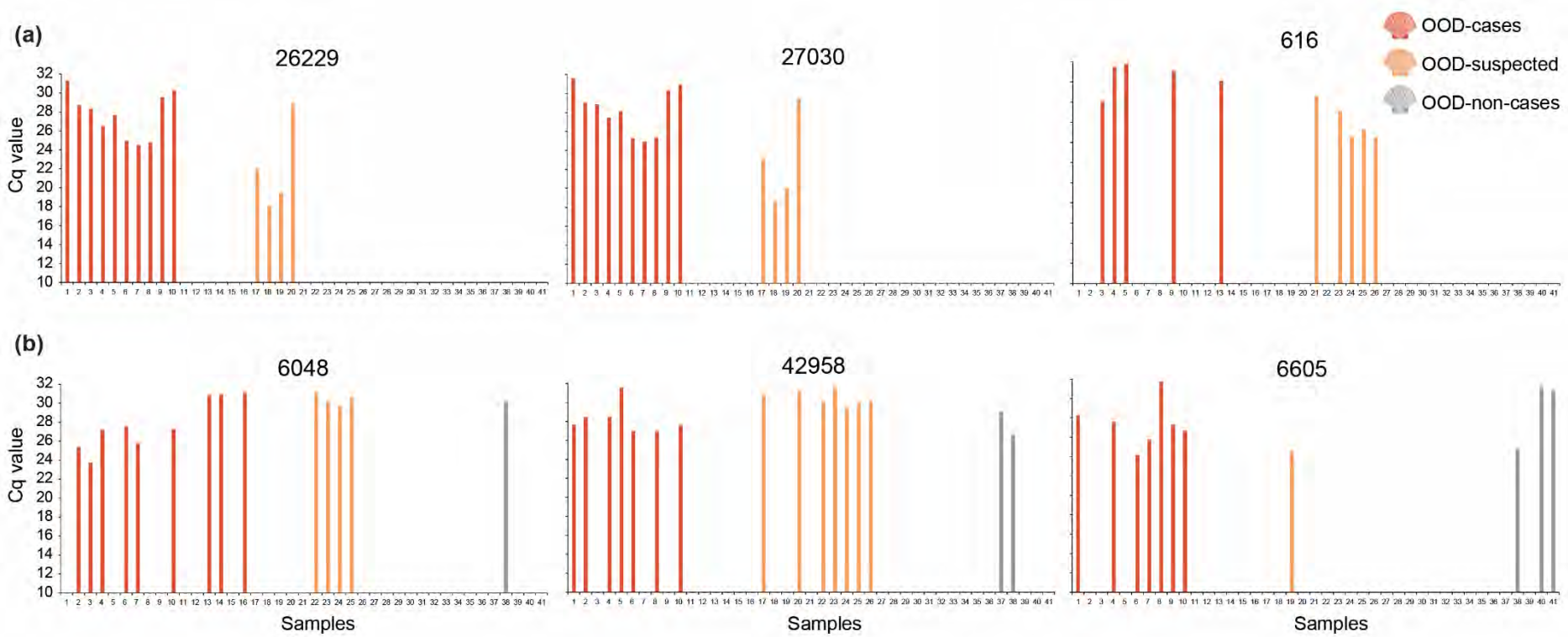

Figure 10. Abundance of nucleotide sequences potentially associated with OOD in *Pinctada maxima*. Contigs-of-interest analysed by qPCR were selected based on the next-generation sequencing (NGS) data. This figure shows examples of contigs-of-interest with exclusive **(a)** or higher **(b)** prevalence in OOD-cases and OOD-suspected oysters relative to non-cases. Red bars indicate OOD-cases (samples 1-16), orange bars represent OOD-suspected oysters (samples 17-26), and grey bars indicate non-affected control oysters (OOD-non-cases) (samples 27-41). Note that the lower the Cq value, the higher the contig abundance.

The remainder of the 86 original contigs-of-interest analysed (69; 80%) did not show any clear association with OOD. These contigs were either similarly abundant across all sets of samples (OOD-cases, OOD-suspected and OOD-non-cases), or the differences in abundance observed between samples were not clear enough to differentiate OOD-cases/OOD-suspected oysters from the non-affected group (OOD-non-case) (Fig. 11).

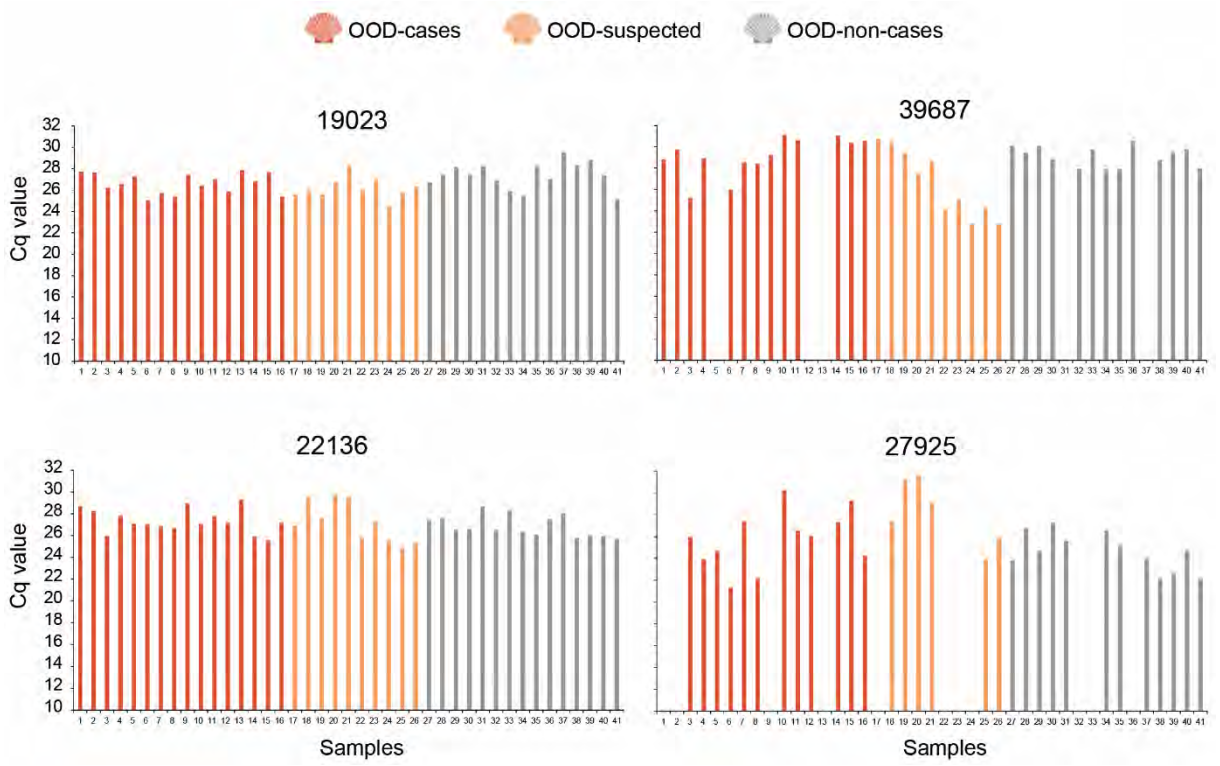

Figure 11. Examples of contigs-of-interest with no clear association with OOD in *Pinctada maxima*. Contigs-of-interest analysed by qPCR were selected based on the next-generation sequencing (NGS) data. Red bars indicate OOD-cases (samples 1-16), orange bars represent OOD-suspected oysters (samples 17-26), and grey bars indicate non-affected control oysters (OOD-non-cases) (samples 27-41). Note that the lower the Cq value, the higher the contig abundance.

An additional set of 8 OOD-non-cases from a separate location were analysed by qPCR at a later stage to bolster the number and geographical distribution of controls. The inclusion of these control samples allowed the further refinement of the list of contigs-of-interest. Primers for 12 of the 17 contigs-of-interest amplified products in more than two of the additional non-affected

control samples. Hence, these 12 contigs-of-interest were excluded from further analysis, reducing the final list of contigs-of-interest from 17 to 5 that were strongly indicative of OOD. These were: contigs 21561, 27014, 27030, 616 and 2830 (Table 5, grey boxes).

In addition to the individual contigs that showed high prevalence in OOD-cases, there were various combinations of two contigs-of-interest that identified up to 75% of OOD-cases and none (or less than 10%) of the non-affected control oysters (OOD-non-cases). These combinations also identified a substantial proportion (20-60%) of oysters that were suspected of suffering from the disease but lacked histopathological confirmation of OOD (OOD-suspected). Examples of these combinations include 21561 + 27014, 21561 + 616 and 27014 + 616 (Fig. 12).

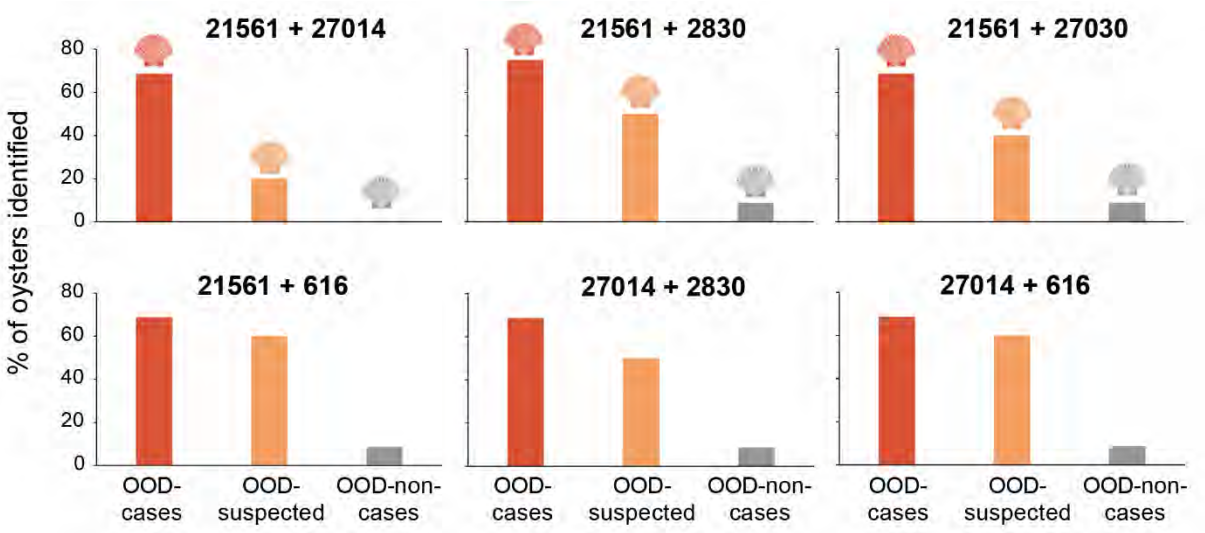

Figure 12. Percentage of each sample group (OOD-cases, n = 16; OOD-suspected, n = 10; OOD-non-cases, n = 23) identified by pairwise combinations of contigs-of-interest.

Table 5. List of the 17 nucleotide sequences potentially associated with OOD. These sequences were investigated by qPCR and found at higher abundance in OOD-cases. Fold-changes (FC) were calculated for both NGS (1) and qPCR (2) analyses from the mean abundance values in OOD-affected oysters (OOD-cases for NGS and OOD-cases + OOD-suspected for qPCR) relative to non-affected oysters (OOD-non-cases). Grey boxes highlight the final list of 5 contigs-of-interest (in bold) selected as indicative of OOD.

Contig #	Sequencing approach	BLASTn			BLASTx			NCBI Conserved Domains	FC
		ID Group	E-value	Bit score	ID Group	E-value	Bit score		
21561	Poly-A	Amphipod	2.00E-14	91.6	Archae	1.4	39.7	No domain	(1) 83.63 / (2) 136.43
26229	Poly-A	Amphipod	6.00E-32	150	Bacteria	7	38.9	No domain	(1) 154.83 / (2) 2824.6
27014	Poly-A	No ID			No ID			No domain	(1) 48.72 / (2) 56.77
27030	Poly-A	No ID			No ID			No domain	(1) 75.42 / (2) 1843.80
27036	Poly-A	No ID			Fungus	9.7	37.7	No domain	(1) 30.47 / (2) 43.83
4203	Poly-A	No ID			Oyster	1.00E-59	223	No domain	(1) 30.14 / (2) 1.43
2830	Total-RNA	No ID			No ID			No domain	(1) 49.05 / (2) 2.94
6048	Total-RNA	No ID			No ID			No domain	(1) 63.47 / (2) 140.17
43053	Total-RNA	No ID			Fish	1.00E-14	85.9	ANK superfamily - ankyrin repeats	(1) 36.46 / (2) 109.26
18143	Total-RNA	No ID			Oyster	8.00E-13	69.7	No domain	(1) 361.24 / (2) 113.17
42958	Total-RNA	No ID			No ID			No domain	(1) 65.84 / (2) 5.81
42653	Total-RNA	No ID			Oyster	8E-146	466	No domain	(1) 36.29 / (2) 1.85
616	Total-RNA	No ID			Bacteria	2.60E-01	43.9	BAR-domain superfamily	(1) 181.45 / (2) 7.25
6605	Total-RNA	No ID			No ID			No domain	(1) 300.66 / (2) 6.81
20870	Total-RNA	No ID			No ID			No domain	(1) 59.71 / (2) 7.47
42397	Total-RNA	No ID			No ID			No domain	(1) 39.22 / (2) 2.70
18888	Total-RNA	No ID			No ID			No domain	(1) 578.39 / (2) 65.98

Multivariate analyses (non-metric multidimensional scaling, NMDS), in which abundance data for the most informative contigs-of-interest were combined into a single plot, also showed a clear delineation between OOD-cases and OOD-non-cases. NMDS plot differentiated OOD-cases and OOD-non-cases into two distinct clusters, with no overlap between ordinates (Fig. 13a) Interestingly, the cluster formed by OOD-suspected oysters overlapped and was intermediate between the OOD-cases and OOD-non-cases clusters (Fig. 13b). It was also apparent that the abundance of contigs-of-interest was far more variable in OOD-cases and OOD-suspected oysters than in OOD-non-cases. This is reflected by the far broader spatial separation of ordinates for OOD-cases and OOD-suspected oysters in NMDS plots (Fig. 13). This is consistent with highly dynamic and rapid changes in contig abundance during the course of OOD and may explain why none of the contigs-of-interest identified all OOD-cases.

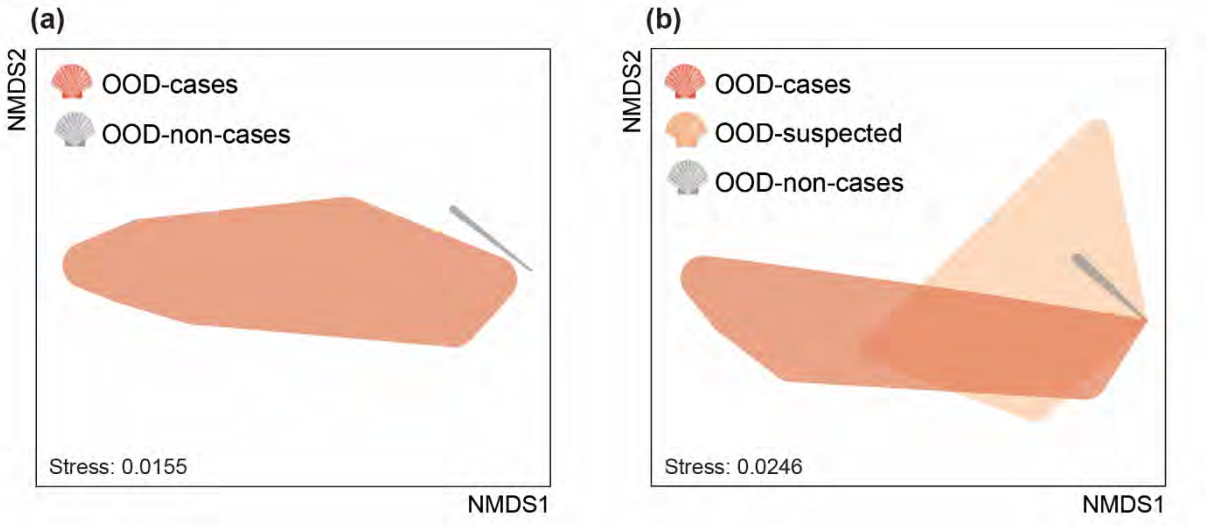

Figure 13. NMDS plots of qPCR abundance data for the most informative contigs-of-interest in OOD-cases (n = 16), OOD-non-cases (non-affected controls; n = 23) and OOD-suspected oysters (n = 10). **(a)** Comparison of OOD-cases and OOD-non-cases. **(b)** Comparison of all 3 groups. Shaded areas show distinct clusters of data points.

The sample sets tested by qPCR were derived from a variety of different locations. This negated the possibility that these results simply reflect geographic differences in normal oyster

microbiota. For instance, one of the OOD-suspected sample sets (samples 22-26 in Figs. 10 and 11) came from the same location that was used previously to collect non-affected oysters (OOD-non-cases) (samples 27-36 in Figs. 10 and 11), but after a mass mortality event was reported at that location. Despite their capacity to differentiate between OOD-cases and OOD-suspected sample sets from non-affected controls (OOD-non-cases), none of the contigs-of-interest identified all OOD-cases.

5.3 Time course analysis of contig abundance

Non-technical summary: qPCR was used to measure the abundance of a refined list of 17 contigs-of-interest in 182 samples from a longitudinal study. The longitudinal study sampled oysters throughout a single high mortality event. This analysis allowed the abundance of particular contigs-of-interest to be correlated with increasing rates of cumulative mortality. The abundance of two of the contigs-of-interest that the previous qPCR analysis had shown to be associated with the presence of OOD was significantly correlated with cumulative mortality. These two contigs-of-interest became more abundant as mortality rates increased.

182 samples from a longitudinal study were analysed by qPCR. Samples were collected over time from four sites that were experiencing abnormally high mortalities. The four sites were within a 5km radius in a single embayment, and were sampled periodically over the course of a mass mortality event. Samples were collected for qPCR analysis and mortality was assessed at each time point. The abundance of the refined list of 17 contigs-of-interest was evaluated in samples from this longitudinal study following the same protocol used in the previous qPCR analyses.

The abundance of some of the contigs-of-interest in oysters from the longitudinal study was significantly correlated with increasing mortality. The abundance of four contigs-of-interest (6605, 2830, 42397 and 616) was significantly correlated ($p < 0.05$) with cumulative mortality when data from all oyster panels, sites and sampling dates were combined (Fig. 14). In this analysis, zeros for cumulative mortality and contig abundance were removed from the datasets to facilitate the visualisation of data distribution. Despite the statistically significant correlations between mortality

and contig abundance for some of the contigs-of-interest, there was also substantial variability in the data. The absolute abundance (Cq values) of two contigs-of-interest (6605 and 2830) was significantly correlated with mortality, such that higher absolute abundance of the contigs was associated with higher mortality rates (Note: The lower the Cq value, the higher the contig abundance. This is the reason for the negative correlation coefficients [R = -0.47 and -0.26] observed in Fig. 14a). Similarly, a strong correlation between relative abundance and mortality was observed for two other contigs (42397 and 616), in that increasing mortality was also associated with increasing contig abundance (Fig. 14b). The association between contig abundance and mortality was particularly evident for contig 6605 where the regression had a substantial slope, a high correlation coefficient (R = -0.47) and was highly significant (p = 0.001). In this context, the abundance of contig 6605 was substantially predictive of mortality.

An association between contig abundance and mortality was also evident when specific panels within a site were examined over time, and these analyses were less prone to variability in the data. For example, the abundance of contigs 2830 and 6605 increased (lower Cq values) in line with mortality over time in two panels (panels E31 and E38) from an affected site (Fig. 15). The highest abundances for each of these contigs were observed on the dates when cumulative mortality approached 100% (2nd October).

Of the four contigs-of-interest that showed a significant correlation with mortality, two were also included in the list of five contigs-of-interest that previous work had deemed to be indicative of OOD. Contigs 616 and 2830 were found at much higher abundance in OOD-cases originating from numerous geographical locations and their abundance was significantly correlated with mortality. As such, these two nucleotide sequences could be used as both predictive of disease and mortality. The contig-of-interest that had the strongest correlation with mortality (6605) was not included in the final refined list of 5 contigs-of-interest only because it was detected in some non-cases in addition to numerous OOD-cases and OOD-suspected oysters.

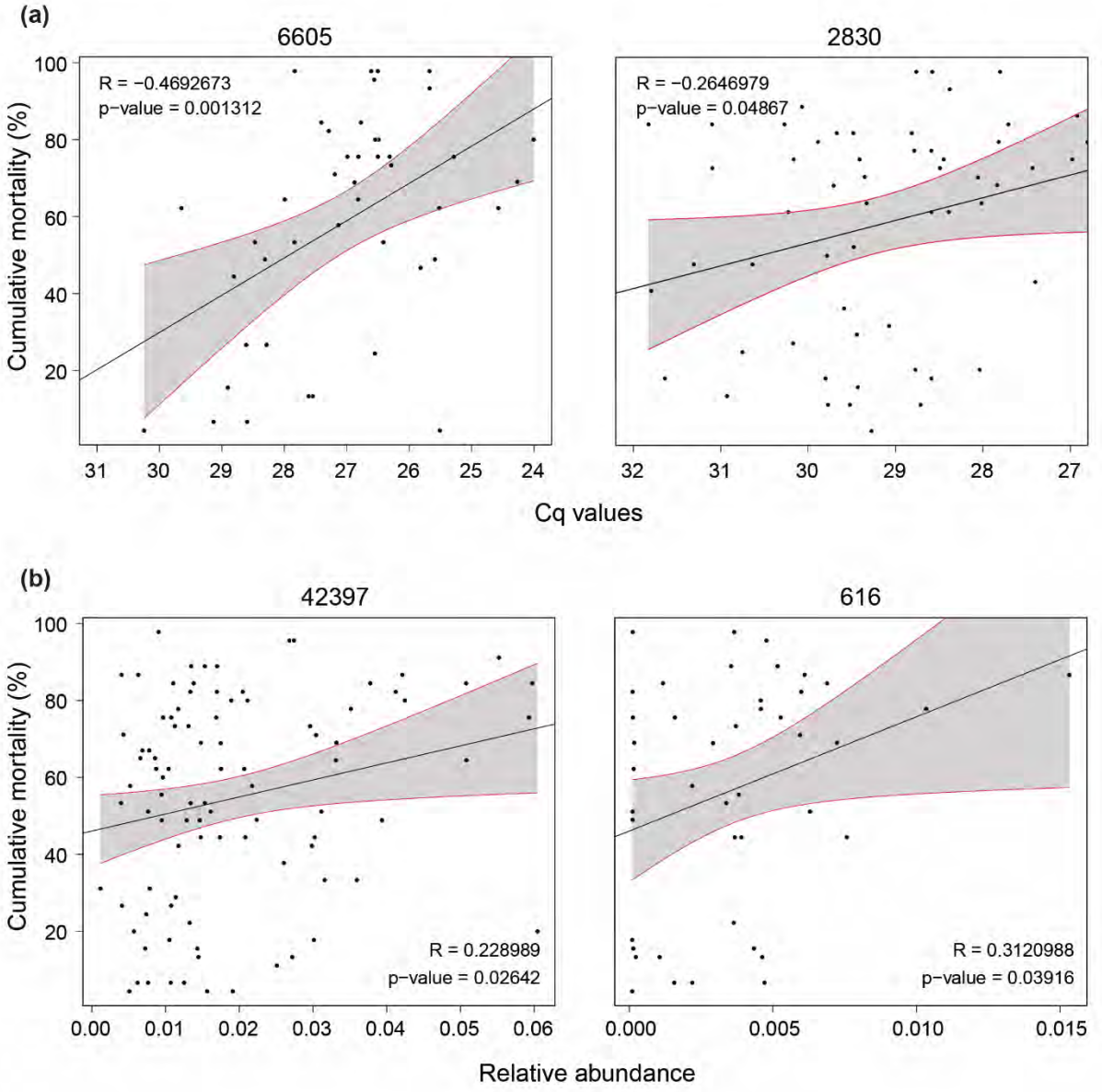

Figure 14. Correlations between the abundance of contigs-of-interest and cumulative mortality among oysters from the longitudinal sampling experiment. Linear regression (black line) with confidence intervals (95%; red lines) of absolute abundance **(a)** and relative abundance **(b)** plotted against cumulative mortality in panels from which oysters were taken during the longitudinal study. Regression lines in **(a)** show negative correlation coefficient because lower Cq values represent higher levels of expression (note that the x-axis showing Cq values is inverted in graphs).

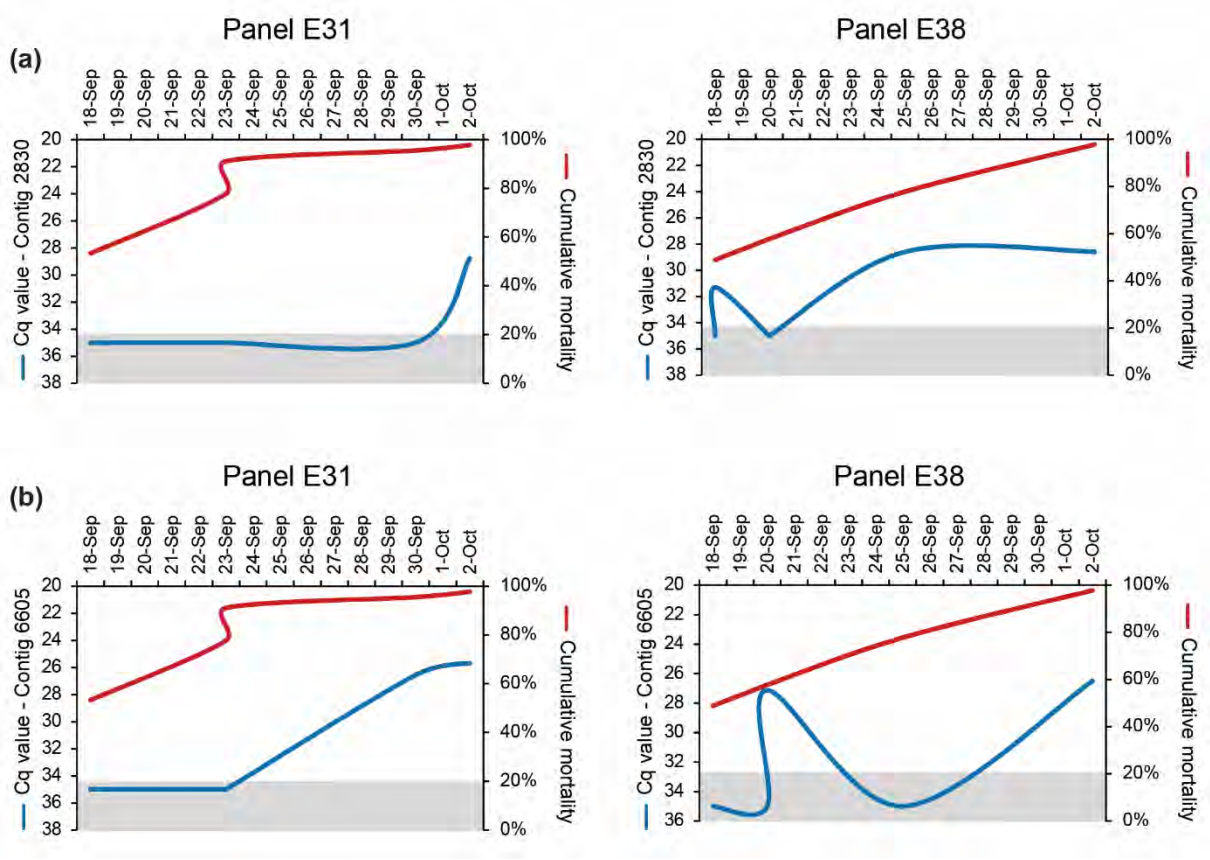

Figure 15. Association between contig abundance and cumulative mortality in single oyster panels from the longitudinal study. Increases in mortality were accompanied by increases in the abundance of contigs 2830 **(a)** and 6605 **(b)** in two panels from the same site. Red lines represent cumulative mortality and blue lines are contig abundance. Areas in grey indicate baseline mortality in oyster panels (≤ 20%). Note that the lower the Cq value, the higher the contig abundance (the primary y-axis showing Cq values is inverted in these graphs for ease of comparison).

5.4 Transmission electron microscopy analysis

Non-technical summary: Electron microscopy did not detect any viruses in tissues from OOD-cases. However, there was clear evidence for the presence of at least one Chlamydia-like organism and a number of other intracellular bacteria. Although present in multiple OOD-cases, the relationship of these Chlamydia-like organisms to OOD has been tested in a previous FRDC project and was found to be inconclusive. We also identified a number of intracellular bacteria in our NGS datasets. However, the contigs representing those bacteria did not differ in abundance between OOD-cases and non-cases, and so were not clearly associated with OOD.

The analysis of 50 negatives from OOD-cases found no evidence of viruses. However, there are a number of prokaryotes visible on many of the negatives. The most common is a circular or oval Chlamydia-like organism of variable size (544 ± 133 nm diameter, n = 20) (Fig. 16a). It exhibits a double trilaminar cell wall with an electron lucent space between, in total about 22 nm thick. The cells sometimes exhibit blebbing or an extension (Fig. 16b). Though superficially similar to mitochondria because of the presence of variably laminar elements in the internal cytoplasm, they do appear distinctly different alongside mitochondria (Fig.16c).

The second type is a mollicute-like dense form also lying either directly in the host cytoplasm or in a vacuole, with a trilaminar membrane separated from a dense core and measuring 175 ± 61 nm (n = 18) (Figs. 17a and 17b).

A much smaller (60-100 nm) mollicute clustered in a vacuole was found on negatives obtained from one grid (Fig. 17c), and one negative (C941) shows a mass of Mycoplasma-like procaryotes associated with a degenerate nucleus (Fig. 17d). Unfortunately, there is no other image to put his plate in context.

Rickettsia and Chlamydia-like bodies have been reported from bivalves on numerous occasions, but mycoplasmas and mollicute-like organisms are rare in the literature. One reason may be that Rickettsia and Chlamydia are commonly reported in membrane bound cytoplasmic vacuoles which stain basophilic on light microscope sections (Li & Wu 2004; Sun & Wu 2004), while mollicute-like procaryotes tend not to be visible on light microscope sections.

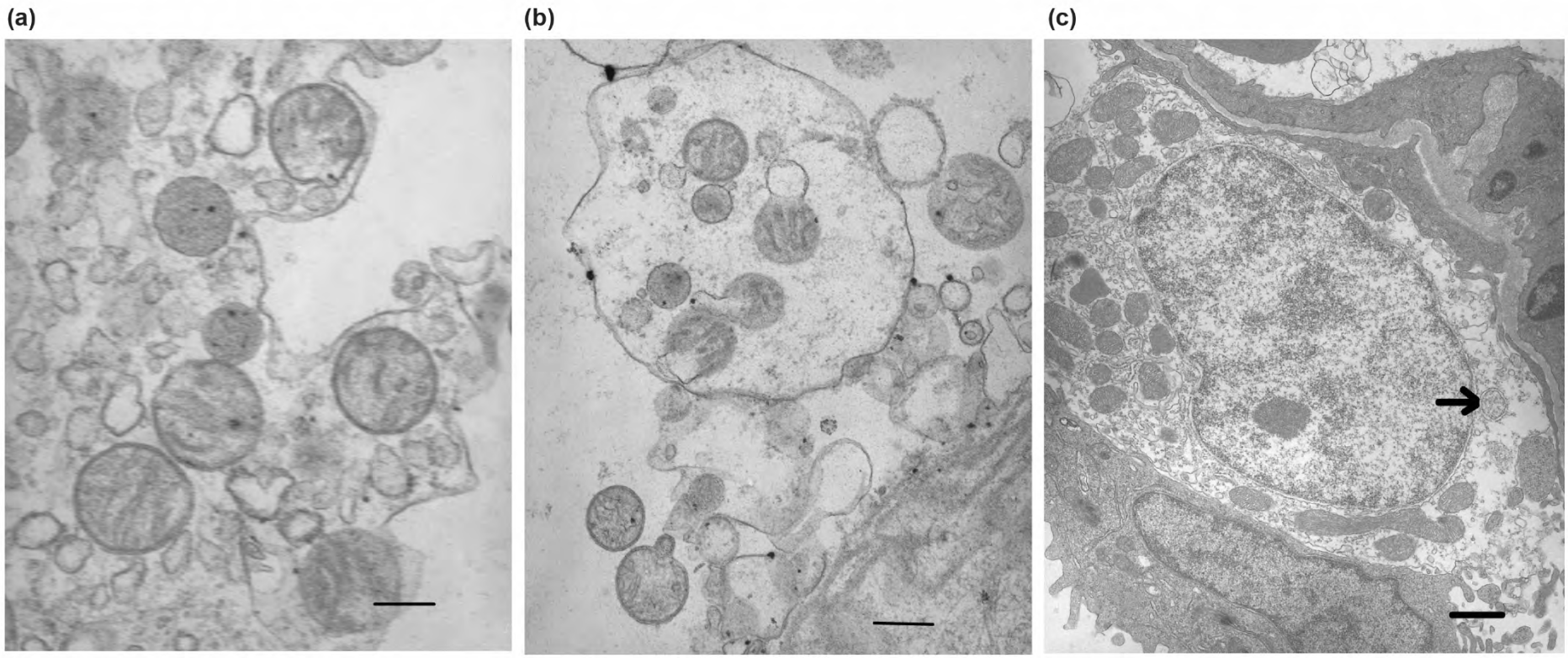

Figure 16. Transmission Electron Microscopy micrographs showing a Chlamydia-like organism in tissues from OOD-cases. **(a)** Group of mollicute-like procaryotes in host cell (Negative C925). Scale bar = 300 nm. **(b)** Mollicute-like procaryotes, including a number with blebs or projections (Negative C926). Scale bar = 200 nm. **(c)** Host cell rich in mitochondria with mollicute-like prokaryote near nucleus (arrow) (Negative C956). Scale bar = 1200 nm.

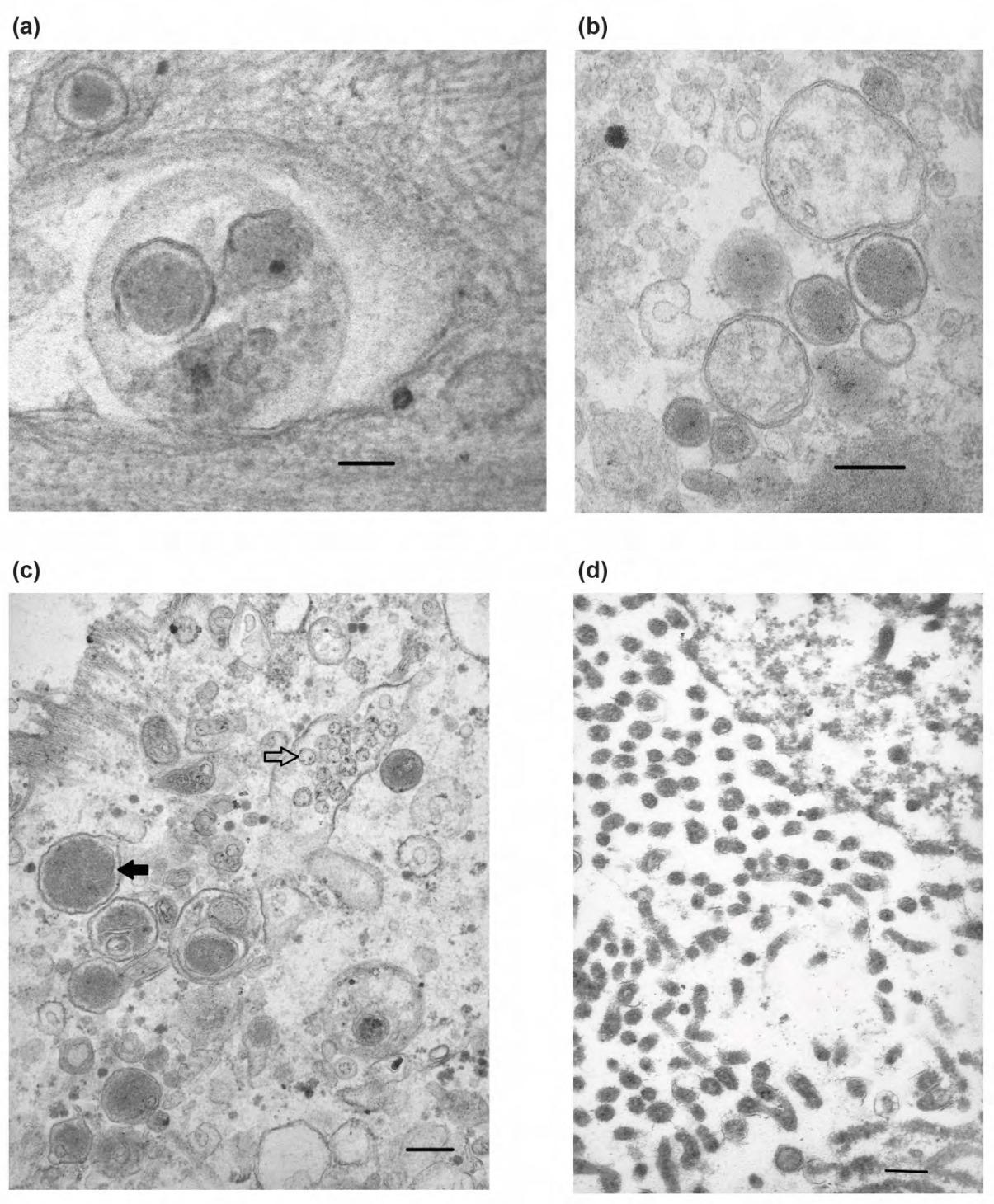

Figure 17. Transmission Electron Microscopy micrographs showing mollicute-like forms in tissues from OOD-cases. **(a)** Dense form of mollicute-like prokaryote, one in vacuole, one in host tissue (Negative C928). Scale bar = 80 nm. **(b)** Both thin and dense form of mollicute-like prokaryote (Negative C936). Scale bar = 300 nm. **(c)** Small mollicutes in vacuole (open arrow) and dense form (solid arrow) (Negative 937). Scale bar = 250 nm. **(d)** Mycoplasma-like bodies (Negative C941). Scale bar = 300 nm.

A Rickettsia-like prokaryote was reported in aquaculture pearl oyster *P. maxima* from China (Wu *et al.* 2001; Wu & Pan 1999) and associated with mortality of 5 month old oysters. However, rickettsia cells were visible in histological sections. The organisms are described as extremely pleomorphic and measure 967 × 551 nm, existing as large cell variant and an electron dense small cell variant (Wu & Pan 1999).

The Chlamydia-like prokaryote is very similar in appearance to ultrastructure of mammalian Chlamydia with blebbing or budding (Beatty *et al.* 1995; Kuo & Chi 1987; Moulder 1991), and to Rickettsia figured in Anderson *et al.* (1965). They also resemble the Chlamydia-like reticulate bodies illustrated by Renault and Cochennec (1995) in their figure 6a-b, from *Crassostrea gigas* from the French Atlantic coast. The second type seen in the negatives from DAFWA, with an electron dense core separated from the membrane by an electron translucent space, resembles the electron dense round to fusiform elementary bodies also described from *Crassostrea gigas* by Renault and Cochennec (1995) and by Comps and Tigé (1999) in the mussel *Mytilus galloprovinciallis*. Whether the reticulate and condensed forms are stages of the same organism is unknown. Chlamydiae modify the outer membranes of their host cells (Moulder 1991).

The third type (60-100 nm diameter) appear to be small procaryotes in a vacuole. They were only observed in one grid, though several negatives of the same section exist. Finally, the fourth type is very similar to the intracytoplasmic infections reported by Molloy *et al.* (2001) from the bivalve *Dreissena* sp. in Greece, but also very similar to species of Mycoplasma (Anderson *et al.* 1965; Biberfeld & Biberfeld 1970; Wilson & Collier 1976). Mycoplasmas disrupt cell membranes, causing oxidative damage and disrupting Cl and K channels in plasma membranes (Razin *et al.* 1998).

In summary, there is evidence on the extant micrographs of at least one chlamydia-like organism and this is in accord with the previous FRDC report (Crockford & Jones 2012). However, its relationship to OOD is inconclusive. The presence of other mycoplasmas and mollicute-like procaryotes is also indicated.

The NGS analysis of OOD-cases and non-cases in the current project also identified mycoplasma- and Rickettsia-like organisms. However, subsequent differential abundance analysis did not find any significant difference in the abundance of contigs representing these organisms between OOD-cases and non-cases. As such, they appear to normal microbiota of pearl oysters.

INDUSTRY ENGAGEMENT

This project was a collaborative effort between researchers and industry. Throughout the project, we have been in contact with all of our relevant industry partners: Pearl Producers Association (PPA), Paspaley Pearls, Cygnet Bay Pearls, Clipper Pearls and Tenerife/Norwest Pearls. Such interactions were primarily focused on discussions on sampling strategies, the progress of the project and directions for future research. In this context, essential tasks, such as decisions on modifications of the research plan and sample collection were shared between all collaborators based on our relative expertise. Industry partners were also responsible for much of the sample collection required for the project.

The participation of our industry partners was particularly relevant with regard to advice on mortality events, the identification of potentially OOD-affected oysters, and their direct involvement in the extensive sampling effort. They provided substantial in-kind and logistical support in the form of access to their oyster farms, boat time and on-site workers to assess oyster leases and perform tissue sampling for histopathological and molecular analyses.

Contact with our industry partners has been through face-to-face and teleconference meetings, email consultations and occasional site visits for staff training. The major meetings held between researchers and industry partners since the start of this project and the topics discussed, as well as collaborative sampling work, are listed below. This list is chronological based on the Milestone Progress Report (MPR) in which the interactions were detailed.

Date: 15 August, 2013 (reported in MPR #1)

- **Media release:** Relevant industry partners (PPA, Paspaley Pearls, Cygnet Bay Pearls and Clipper Pearls) were contacted to discuss the draft media release describing the project, which was subsequently finalised and released (Appendix 7).

- **Sampling strategy and protocols:** A face-to-face meeting (Broome, March 28, 2013) and teleconferences were held with PPA and industry to develop project objectives and sampling regimes and to finalise sampling protocols.
- **Planning of staff training and sample collection:** There was also extensive discussion with staff from the Kimberly Marine Research Station (KMRS; Cygnet Bay) regarding sampling regimes and their contribution to sampling. Prof. Raftos and Dr. Snow planned a trip to KMRS to finalise those plans and train staff.

Date: 30 September, 2013 (reported in MPR #2)

- **Staff training and sample collection:** Prof. Raftos and Dr. Snow travelled to Cygnet Bay in late August 2013 to train staff from the Kimberly Marine Research Centre (KMRS) in sampling techniques and to finalise details of the sampling strategy. This trip enabled the direct interaction between the researchers and the industry partners that were involved in the initial sampling of oyster tissues for identification of potential pathogens. The recipients of that training were Dr. Erin McGinty (Research Manager at KMRS) and James Brown (General Manager, Cygnet Bay Pearls). Dr. McGinty already had extensive experience in tissue sampling and processing tissue from pearl oysters (having completed her PhD in this area), and James Brown is an expert on pearl oyster anatomy and husbandry. During the training sessions, protocols for an additional round of sample collection, which was undertaken by KMRS staff from September 2013 onwards, was finalised. That additional round of sampling was commenced during the visit, and plans were put in place for further sample collection, storage and shipment.
- **Purchase of reagents and consumables:** During the trip to KMRS, Prof. Raftos and Dr. Show identified the consumables (collection tubes, dissecting instruments, etc.) and reagents (RNAlater®, fixatives, etc.) that were needed for sample collection. Those items were ordered and shipped to Fisheries WA office in Broome for collection by KMRS staff. Later in the project, complete sampling kits were assembled and shipped to all of the industry partners.

- **Meeting with industry partners to finalise sampling regime:** Teleconference meetings with industry partners were held to discuss the sampling protocol that has been developed during the visit to KMRS. It also allowed the establishment of a system for storage and shipment of samples to Perth and Sydney for analysis.
- **Staff recruitment:** Staff recruitment was also finalised. Dr. Kelli Anderson was originally nominated to continue in the postdoctoral position funded by this project. Dr. Anderson had worked on the project before funding became available from FRDC. However, Dr. Anderson took up another (full-time, permanent) position before funds were made available for 2013/002. As a result, the position was offered to Dr. David Jones.

Date: 13 January, 2014 (reported in MPR #4)

- **Longitudinal sampling study:** An extensive cycle of sample collection was undertaken by KMRS staff from September to December 2013. Samples were collected from five sites on the Dampier Peninsula, WA (longitudinal sampling study). A total of 659 samples were collected and stored for molecular and/or histopathology analyses. After sampling was completed, samples were shipped to Fisheries WA for histopathological analysis and storage prior to molecular analysis. During this sampling, mortality data were also recorded by KMRS staff.
- **Revision of the sampling protocol:** Following advice of the Aquatic Animal Health (AAH) Subprogram committee, we consulted a professional epidemiologist regarding our sampling regime. Dr. Ben Madin, from AusVet, has extensive experience with OOD. Dr. Madin provided assistance refining our sampling regime based on the formal case definition of the disease. He also confirmed that our experimental design and sampling protocols were appropriate.
- **Sampling for next-generation sequencing completed:** Sample collection from oysters with the clinical signs of OOD to be used in NGS was completed with the assistance of industry. Sampling for this first phase of the project was opportunistic. That is, samples were collected at

the time and location where industry reported ongoing unexplained mortalities that they suspected were caused by OOD. Sampling was performed by farmers and researchers involved in the project.

Date: 30 September, 2014 (reported in MPR #5)

- **Meetings with industry partners to discuss progress of the project:** Two teleconferences were held with key members from the commercial partners, the industry association, and all chief investigators to discuss the progress, direction and logistics of the project. It included representatives of Paspaley Pearls, Clipper Pearls, Cygnet Bay Pearls, Pearl Producers Association, Fisheries WA, New Zealand Ministry for Primary Industries (Manatū Ahu Matua) and Macquarie University. To ensure all stakeholders and chief investigators were satisfied with the project direction, strategy and expected outcomes, these two teleconferences gave all participants the opportunity to voice concerns and make suggestions. During the first meeting (held in February 2014), we spoke in detail about the completion of initial sampling, histopathological analysis of samples, analysis of existing NGS data, the selection of samples for NGS, and strategy for establishment of bioinformatic workflows. The second teleconference was held in September 2014 and discussed commercial-in-confidence issues regarding sampling and opportunistic sampling of OOD-cases upon the appearance of an outbreak. An update on the progress of the project and timeline for the ongoing work were also provided by the chief investigators.
- **Selection of samples for next-generation sequencing:** A third meeting has been held in Perth, WA. It was attended by Prof. Raftos, Dr. Jones, Dr. Snow and staff from the Aquatic Animal Health Unit - Department of Fisheries WA. The outcome of this meeting was the selection of the most appropriate samples for use in the second round of NGS. Sample selection was based on the presence of gross and histopathological signs of OOD according to the case definition of the disease. A letter from Department of Fisheries WA confirming their advice on the samples to be used for NGS is attached (Appendix 8). Whilst in Perth, Prof. Raftos, Dr. Jones and Dr. Snow also

developed a robust workflow for processing the NGS data to discover potential pathogens involved in OOD.

- **Preparation of sampling kits:** Due to potential issues with the transport of dangerous goods (*i.e.* formaldehyde), sample collection kits (with instructions) were put together by Dr. D. Jones and Prof. Raftos. These kits were sent to all pearl companies in preparation for any occurrence of OOD. They included all reagents and equipment necessary for sampling and appropriate storage of tissues for both histopathology and genetic analysis (allowing the sampling of 20-50 oysters per kit). This initiative enabled rapid sampling and minimised delays regarding acquisition of sampling equipment in remote farming locations.

Date: 1 April, 2015 (reported in MPR #6)

- **Meeting with industry partners to discuss progress of the project:** A teleconference was held with stakeholders in March 2015 to discuss the progress, direction and logistics of the project. All key industry partners (Paspaley Pearls, Clipper Pearls, Cygnet Bay Pearls and Pearl Producers Association) and research representatives (Department of Fisheries WA - Aquatic Animal Health Unit, Macquarie University) attended. This teleconference was used as a bi-annual check to ensure all stakeholders and chief investigators were satisfied with the direction, strategy and expected outcomes of the project to date. It also gave all participants the opportunity to voice concerns and make suggestions. During this meeting, we spoke in detail about concerns regarding the (i) clinical definition and diagnosis of OOD, (ii) opportunistic sampling of OOD-cases and matching controls from the same geographical location or in close proximity to OOD sites, (iii) an audit of samples already collected for qPCR validation, (iv) options and personnel for the qPCR phase of the project, (v) implications regarding the completion of Dr. David Jones contract, (vi) inclusion of the locations and dates of all sample collection in a confidential Addendum to MPR #6, and (vii) the inclusion of Roger Barnard from Tenerife/Norwest Pearls as a stakeholder on this project due to his heavy involvement in the initial 2006/7 Exmouth Gulf OOD outbreak and his

position to provide additional samples. A detailed update on the progress of the project and timeline for the ongoing work was also provided by the chief investigators.

Date: 23 June, 2015 (reported in the response to comments on the MPR #6)

- **Communication of results at the AAHS conference:** Participation of Dr. David Jones in the AAHS conference in Cairns, QLD. Dr. Jones attended the conference to present the results of our next-generation sequencing.

Date: 4 March, 2016 (reported in the Revised Research Plan for FRDC 2013/002)

- **Revision of the specific objectives and plans of action of the project:** Following advice of the AAHS committee, the specific objectives and plan of action for the project were revised. A teleconference was held with all stakeholders on 4 March, 2016 to gain their approval for the revised research plan. We included in this revised research plan: (i) analysis of the longitudinal samples with the view of narrowing the range of potential pathogens identified to date; (ii) re-analysis of the NGS data to obtain larger contigs, which may also be beneficial towards narrowing the range of potential pathogens; (iii) electron microscopy analysis of tissues from OOD-impacted oysters in an effort to visually identify any potential infectious agents in the tissues; and (iv) a commitment to hold a workshop attended by industry representatives and relevant experts in the field to describe the findings of the current projects and discuss future options for OOD-research. These revisions to the research plan were universally accepted by our industry stakeholders.

Date: 1 October, 2016 (reported in MPR #7)

- **Reactivation of the project:** The project was reactivated after a 11-month suspension (since April 2015) awaiting the approval of MPR #6 and acceptance of the revised research plan. The recommencement of the project on 14 March 2016 was approved by our industry partners and research representatives.

- **Meeting with stakeholders to discuss plan of action:** A teleconference was held with stakeholders in March 2016, a week before the official reactivation of the project. The majority of the key industry partners (Paspaley Pearls, Pearl Producers Association and Tenerife/Norwest Pearls) and project researchers (New Zealand Ministry for Primary Industries and Macquarie University) attended. The meeting was convened to seek approval for a plan of action to complete the project within the framework of a revised research plan (subsequently accepted by FRDC). A detailed timeline was presented and approved by all representatives.
- **Staff recruitment:** The March 2016 meeting also approved the appointment of Dr. Priscila Gonçalves as the new post-doctoral researcher of the project. She was engaged to undertake the relevant molecular work to address each of the objectives specified in the revised research plan, including: (i) qPCR evaluation of contigs-of-interest identified by NGS; (ii) reanalysis of existing NGS and further qPCR evaluation; and (iii) comprehensive screening by qPCR of the final refined set of contigs-of-interest across a large suite of samples from a longitudinal study (geographical and temporal range).
- **Periodic summary of the progress:** After the reactivation of the project, we sent reports (plain-English and technical) to the key industry partners and research representatives as steps of the experimental work were concluded. From March to October 2016, we disseminated three reports on lab work and one report on the NGS reanalysis to all key industry partners and research representatives.
- **Timing and planning of workshop to discuss extension of results to date:** We have also worked with the Pearl Producers Association (PPA) regarding the timing and format of the proposed workshop. Aaron Irving (PPA) and Dr. David Mills (Paspaley Pearls) suggested that the workshop to present our results and discuss future directions should be held in Cairns (QLD) to coincide with the next Aquatic Animal Health conference sponsored by FRDC. A satellite meeting around the Cairns conference would allow input from a broad range of the experts who attend the conference and would substantially decrease expense relative to a stand-alone meeting. PPA put

this plan to all of its members. We will start consulting with PPA and AAHS regarding potential formats for the workshop in early 2017.

CONCLUSIONS, PERSPECTIVES AND IMPLICATIONS

This project has identified a number of nucleotide sequences (termed contigs-of-interest in this report) that are associated with OOD in terms of their prevalence among OOD-cases relative to non-cases. The abundance of some of these contigs-of-interest also exhibited statistically significant correlations with increasing mortality during a mass mortality event. These characteristics mean that our final refined list of contigs-of-interest represents an extremely valuable resource for further studies of OOD and some of our contigs-of-interest may prove to be useful predictors of mortality.

None of the contigs-of-interest that we identified is closely related to any known infectious agent and none of the contigs are capable of identifying all OOD-cases. In that context, they may have limited value used by themselves in routine diagnostic tests. However, further work might improve the frequency with which these contigs-of-interest identify OOD-cases, and the development of technologies for multigene arrays based on these contigs-of-interest may provide a novel and effective diagnostic tool for OOD.

Our inability to identify individual contigs that definitively differentiate between OOD-cases and non-cases must bring into question assumptions that OOD is an infectious disease. At the least, we can say with confidence that our analyses provide no evidence for a virus as the causative agent of OOD. Our NGS data confirmed the ability of this technology to identify viruses. Numerous contigs with strong similarities to known viruses were present in our dataset. However, none of the virus-like contigs differed in abundance between OOD-cases and non-cases, and so were clearly not causative of OOD. The lack of viral involvement in the disease is supported by fresh electron microscopy analyses of tissues from OOD-cases. Again, that TEM analysis did not identify any viral particles in pearl oyster tissue. Moreover, transcriptomic comparisons of OOD-cases and non-cases undertaken by other workers outside this project have not detected any

activation of anti-viral immune responses in OOD-affected oysters. All of this evidence suggests that OOD is not a viral disease.

We are confident that our next-generation sequencing (NGS) approach would have identified any causative infectious agent that was present at high abundance when indicative features of OOD (high mortality, gross signs and tissue oedema) were apparent in oysters. It is unlikely that we rejected nucleotide sequence reads from potential causative agents of OOD in the filtering steps associated with assembling NGS data. Most of the reads that we rejected were present at low abundance and would have been represented by numerous other higher quality counterparts. We have also limited the possibility of not identifying cryptic infectious agents (those that are restricted to a highly localised site of infection within oysters and gain their pathological effects systemically) by sampling a broad range of tissues within each oyster. However, it is possible that a pathological agent might remain external to the oyster and gain its effects through the release of a chemical toxin, which we would not have identified by NGS. Below, we recommend further investigating whether harmful algal biotoxins may cause OOD.

Despite the necessary qualifications to our work, we have identified a number of contigs-of-interest that provide a clear delineation between OOD-affected oysters and non-affected controls, as well as identifying many oysters that were suspected of suffering from OOD (high mortality area and gross signs) but did not show tissue oedema. The lack of sequence similarity between these contigs-of-interest and known pathogens leaves open the possibility that the contigs-of-interest do come from an infectious microbe that causes OOD, but that the microbe represents a previously uncharacterised pathogen. An alternative explanation is that our contigs-of-interest are uncharacterised oyster genes responding to OOD.

We are also confident that the contigs-of-interest that we identified do not simply reflect geographic or temporal differences in the normal microbiota of oysters. The samples that we tested were from a broad range of geographic locations and times, so the patterns of contig abundance that we identified are unlikely to reflect location- or time-specific differences in the normal

microbiota of oysters. In contrast, it is entirely possible that our contigs-of-interest reflect changes in oysters resulting from abiotic disease and morbidity.

RECOMMENDATIONS

1. Conduct a thorough transcriptomic analysis comparing OOD-cases and non-cases to identify the genes that oysters switch on and off in response to OOD. This has a high likelihood of defining the nature of the disease. It is also likely that analysing oyster genes will provide a more effective diagnostic technique for OOD than those sought in the current project.

2. Explore the development of multigene array or high throughput comparative sequencing technologies based on the contigs-of-interest that we identified as a novel and effective way of predictively diagnosing OOD.

3. Safely archive the samples collected in this study so that they can be used to extend the analysis of the contigs-of-interest that we have identified and to investigate transcriptomic differences between oysters.

4. Collect a panel of external shell swabs and water samples during high mortality events to begin searching for an external agent, such as a toxic alga, that might be associated with OOD, and engage with experts in the area of shellfish biotoxins.

5. Continue to explore the possibility that OOD is not an infectious disease and may have a number of causes including environmental factors.

6. Convene a meeting of industry stakeholders to determine industry priorities in this area.

EXTENSION AND ADOPTION

Extension Target	Who was responsible?	Tools used	Performance indicators	Outcomes	Adoption	Impact	Rating
Primary – Pearl Producers Association/Australian pearling industry	Researchers	• Face-to-face and teleconference meetings with PPA and industry to develop project objectives and sampling regimes • Email consultations with PPA and industry to develop media releases • Communication with PPA and industry throughout project to confirm logistics and feasibility of sampling protocols • Reports presented to a project steering committee comprising all relevant industry stakeholders • Development of an online portal so that industry and other stakeholders can access data and analysis • Development and dissemination of a protocol for industry to use in evaluating the efficacy of potential diagnostic markers • Training of industry staff in sampling protocols and implementation of diagnostic tests • Scheduled meetings with PPA and industry to disseminate data and conclusions from the project	• Ongoing industry engagement in project • Successfully complete all scheduled and ad hoc consultations with industry • Uptake by industry of data, conclusions and tests developed during project • Industry staff trained in sampling protocols and sampling successfully completed	• Enhanced industry knowledge and understanding of OOD • Industry use of any diagnostic tests for OOD developed during project • Professional development of industry staff in scientific protocols pertinent to OOD • Ongoing engagement between industry and researchers	35	30	Extreme

Extension Target	Who was responsible?	Tools used	Performance indicators	Outcomes	Adoption	Impact	Rating
Primary – Pearl Oyster Health Working Group	Fisheries WA in consultation with researchers	• Scheduled meetings of Pearl Oyster Health Working Group comprising all relevant government agencies and industry representatives with discussion of outcomes from 2013/002 as major agenda item	Successfully complete all Pearl Oyster Health Working Group agenda items and actions pertaining to 2013/002	All relevant government agencies and industry representatives: • given opportunity to provide input into project development, outcomes and objectives • fully informed project outputs and conclusions • broad understanding of OOD and potential diagnostic tests disseminated to industry and government agencies	40	25	Extreme
Primary – FRDC Aquatic Animal Health Subprogram	• Researchers • AAHS	• Project application process (EOI and draft full applications) • Scheduled milestone reports • FRDC final report	• Successfully develop funded project proposal in consultation with AAHS • On time submission of all milestone reports, and responding to AAHS' comments on draft reports • Completion of FRDC Final Report in consultation with AAHS	Project fully aligned with objectives of AAHS	35	20	High

Extension Target	Who was responsible?	Tools used	Performance indicators	Outcomes	Adoption	Impact	Rating
Primary – Fisheries WA	Researchers	• Develop diagnostic testing protocols • Train Fisheries WA staff in diagnostic testing	• Implementation of diagnostic testing regime	• Development of a diagnostic testing regime available for industry use	25	35	Extreme
Secondary – Other aquaculture industries, government agencies and researchers	Researchers	Present project data at: • FRDC Australasian Conferences on Aquatic Animal Health • Other aquaculture and scientific conferences • Peer reviewed scientific publications • FRDC final report.	• Successfully complete conference and workshop presentations, publications and FRDC final report • Work referenced in other research projects, reports, etc.	• Dissemination of project data and outcomes to the broader aquaculture and scientific communities	31	20	High
Tertiary – general public (particularly in WA and NT)	• Researcher • FRDC	• Media release on 2013/002 and follow up media inquiries • Preparation of plain English "Fisheries Fact Sheets" by Fisheries WA	• Improved perception of pearl oyster industry and collaboration with researchers	• Awareness of aquaculture research within the community	35	15	Medium

PROJECT MATERIALS DEVELOPED

This project represents the most comprehensive molecular resource on the pearl oyster *Pinctada maxima*. It produced an extensive database of oyster nucleotide sequences, as well as a large inventory of tissue samples originating from oysters collected at multiple locations, different life-history stages and different stages of disease or sickness.

Our extensive analysis on the nucleotide sequences associated with the cause of OOD identified five nucleotide sequences that are strongly associated with OOD (contigs 2830, 616, 21561, 27014 and 27030). These sequences were consistently found at higher abundance in OOD-cases relative to healthy controls collected across broad geographical and temporal ranges. The relative abundance of two of the sequences was also positively correlated with cumulative mortality in oyster samples collected from a longitudinal (time-course) sampling experiment (contigs 2830 and 616). An additional sequence (6605) was included in this list because it showed the strongest correlation with mortality. This sequence was detected in some of non-cases in addition to numerous OOD-cases and OOD suspected oysters and so warrants further investigation. The primer pairs designed to detect the abundance of these nucleotides sequences are shown in Table 6.

Table 6. Primer pairs designed in this study to identify oysters highly affected by OOD. Contigs indicated by 1 could be used as predictive of disease, since these sequences were found at far higher abundance in OOD-cases (relative to healthy controls). Contigs indicated by 2 could be used as predictive of mortality, since their abundance was strongly correlated with the progression of the disease.

Contig #	Sequence (Fw/Rv: 5'→ 3')	Amplicon	E[a]	Application
2830	CCTCTTGTTGGTCATTTCCTG TCAACCTTTGCTCCTTTAATCA	99 bp	104%	1 + 2
616	GCTTGCTTGGGAGATCGACA AAGCGACTTGACCTTGGCTGT	129 bp	100%	1 + 2
21561	GTGACCTCACAACGAACCGC CGGAGGGCAAAGCGTGATAG	138 bp	101%	1
27014	TCTGCTAAGTCCGAGGCCAC AAGCGTTCCAGCACATGAGC	71 bp	99%	1
27030	TTCTTTCGACACCTTGCGGC GCACGATGCTACCTGAGTGC	104 bp	95%	1
6605	TCCGACTGAACATCCGGGTTC CATGGTCATTTGGGCCACCC	111 bp	91%	2

[a] E = Amplification efficiency

APPENDICES

Appendix 1. Quality and concentration of RNA samples used for molecular analyses

Appendix 2. Contigs and primer sequences analysed by qPCR.

Appendix 3. Assessment of potential reference genes.

Appendix 4. List of nucleotide sequences (contigs-of-interest) that were found at significantly higher levels in OOD-cases compared to non-cases.

Appendix 5. Abundance profiles of all 86 contigs-of-interest tested by qPCR.

Appendix 6. BLAST results for the 17 contigs-of-interest potentially associated with OOD.

Appendix 7. Media release on the project 2013/002.

Appendix 8. A letter from Department of Fisheries WA confirming their advice on the samples selected to be used for NGS.

Appendix 9. Supporting references (unpublished reports).

Appendices 1-6 are available upon request from FRDC (frdc@frdc.com.au)

Appendix 7. Media release on the project 2013/002.

Media release on 2013/002

Researchers use cutting edge technology to study diseases in Australian pearl oysters

Researchers from Macquarie University in Sydney Australia and the Western Australian Department of Fisheries have joined forces with the Fisheries Research and Development Corporation and the Australian pearl industry to study the causes of disease in pearl oysters. Infectious diseases can limit the productivity of pearling in Australia, or increase production costs for this important aquaculture industry. However, the causes of disease are often unknown. The researchers will use the world's most cutting edge technology – next generation DNA sequencing – to identify the infectious microbes that might be affecting pearl oysters. Professor David Raftos from Macquarie University says "identifying infectious viruses and bacteria is like looking for a needle in a haystack…so you need to use the best possible technology in the search. Next gen sequencing provides that technology. It allows us to search through literally billions of DNA sequences and use them as fingerprints to identify infectious microbes". Dr Mike Snow from the Western Australian Department of Fisheries added that "the end product of this 3-year research project will be the development of highly sensitive diagnostic tests for infectious agents that may cause disease in pearl oysters. Development of these tests is critical to helping regulators and industry work together to maintain a high health status within the important pearl oyster industry".

Appendix 8. A letter from Department of Fisheries WA confirming their advice on the samples selected to be used for NGS.

Government of **Western Australia**
Department of **Fisheries**

Ref: 818/121
30 July 2014

Professor David Raftos
Department of Biological Sciences
Macquarie University
NSW 2109

Dear Professor Raftos

You recently requested advice on obtaining the most appropriate samples suited to the aims of your project "Identifying the cause of Oyster Oedema Disease (OOD) in pearl oysters (*Pinctada maxima*), and developing diagnostic tests for OOD".

As you know, our group has been heavily involved in research surrounding OOD since recognition of the condition in late 2006, including development of a histopathological case definition for the disease as follows:

Affected animals:

- Silver-lipped pearl oysters (*Pinctada maxima)* only
- Dead or dying, with
- (Grossly) mantle retraction, poor adductor muscle function and mild swelling, otherwise normal in appearance, and/or
- (Histology) showing no signs of an inflammatory process or the presence of pathogens
- On a farm on which mortalities have exceeded normal mortalities for the developmental and management stage of the oyster

Early research into OOD had a strong focus on development of histological analysis as a diagnostic tool and as a result few suitable parallel molecular samples were archived by the Department.

Having reviewed our archives and associated histopathological database, we have identified a set of samples (Case number 07-87) from 2007 for which stored tissue is available and histological lesions consistent with Oyster Oedema Disease were noted in parallel samples. Whilst these tissue samples have been stored in long-term -20 storage, should useable nucleic acid be recoverable from them, they would represent the best prospect of identifying a pathogen associated with OOD during the early emergence of the disease.

You also recently provided some contemporary samples for histological evaluation (Case FH-13-63) which comprised samples provided by industry that had reported mortalities due to OOD. Ten (10) each of both affected and unaffected samples were provided and parallel molecular and histological samples taken from the same individual oysters. In these samples, marked oedema was present in the absence of a haemocytic response and oyster oedema disease was diagnosed. These samples are those most suited to the aims of your project that are available. In particular, animals 3,4,6 and 7 of the OOD diagnosed animals were most severely affected and most suited to examination.

/Continued

Over the past few years the Department has received sporadic samples from 3 producers in the Kimberley, reporting OOD. Diagnosis of OOD has, however, not been consistent, often due to the presence of haemocytes and confounding factors (such as presence of bacteria, necrosis, lack of oedema). As a result, the specimens from the two cases identified above represent the most suitable samples for your project that can be obtained.

Yours sincerely

F. Stephens

Dr Fran Stephens
Senior Pathologist
DoF Fish Health Laboratories
South Perth

Appendix 9. Supporting references (unpublished reports).

Research priorities document for the Australian pearl oyster industry prepared for the Pearl Oyster Health Working Group

OYSTER ODEMA DISEASE - RESEARCH PRIORITIES

Prepared by Brian Jones and David Raftos

Preamble

OOD continues to have extremely serious impacts on pearl oyster farming in WA. Although considerable progress has been made in studying the disease, we still do not have sufficient information to be able to establish effective management strategies. In particular, we do not sufficiently understand:

1. the causative agent(s) of OOD
2. the role of environmental stress in causing susceptibility to OOD

Research plan

Ongoing research should address these outstanding questions in an effort to:

- devise a robust diagnostic test for OOD
- identify possible environmental factors that can be ameliorated to decrease the impact of OOD.

To begin to answer these questions, we recommend a one to two year pilot project ($150-300k total) that specifically addresses the following questions:

- what causes OOD?
- how is susceptibility to OOD affected by environmental stress?

Experimental design and analysis

Collect samples from juvenlie oysters, from the time that they leave the hatchery to the time when they start to die from OOD. Simultaneously analyse these samples for:

- OOD pathology (also includes analysis of existing piggyback spat samples)
- potential viral infection (by PCR), or markers of viral infection (virally induced programmed cell death)
- simple markers of "stress" (phenoloxidase and other oxidative stress responses, as well as changes in other components of cells that are known to be affected by environmental stress and infection)
- complex markers of stress and disease (proteomics and transcriptomics)

Then compare all of these data over time to identify factors that are associated with OOD.

Potential problems

Unless we can identify areas known <u>not to be affected by OOD</u>, this experiment lacks effective controls (non-exposed oysters). Studying disease progression over time partially gets around this problem, because the 0-time point (before OOD is apparent in the hatchery) represents a quasi-control.

Outcomes – the project will provide:

- an effective diagnostic test for OOD, or identification of molecular markers of disease
- an understanding of the biological processes in oysters associated with susceptibility to OOD
- essential background data for subsequent research programs looking at disease resistance

Pearl Oyster Health Working Group minutes

Pearl Oyster Health Working Group Meeting
Monday 20 February 2012
3.00pm

Department of Fisheries Head Office
Meeting Room 1

SUMMARY OF PROCEEDINGS

Attendees:

Dr Brian Jones (Chairman)
Mr Clinton Syers (DoF)
Ms Nat Moore (DoF)
Mr Stuart McDowall (DoF)
Ms Pia Carter (DoF)
A/Prof Dr Dave Raftos (Macquarie University)
Dr Kitman Dyrting (NT Fisheries)
Mr Murray Barton (NT Fisheries)
Mr Patrick Moase (Clipper Pearls)
Dr Dave Mills (Paspaley)
Mr James Brown (Cygnet Bay)
Mr Brett McCallum (PPA)
Ms Leigh Taylor (DoF-minutes)

Apologies:

Mr Peter Godfrey (DoF)
Mr Alex Ogg (Clipper Pearls)
Mr Dave Jackson (Tennereef)
Mr Shane O'Donoghue (DoF)
Dr Sam Buchanan (Blue Seas)
Mr Bruce Brown (Cygnet Bay)

1 Welcome / Apologies

Apologies were tabled for Mr P. Godfrey, Mr A. Ogg, Mr D. Jackson, Mr S. O'Donoghue, Dr S. Buchanan and Mr B. Brown.

2 Update on OOD

Dr Jones advised that no formal advice had been received from any pearling licensee regarding OOD. Dr Jones advised that while he was aware of mortalities continuing based on informal conversations with some licensees, industry needed to formally inform the Department of Fisheries (the Department).

Mr Brown advised that Cygnet Bay Pearls were still experiencing mortalities in hatchery produced oysters.

Mr Mills advised that Paspaley was also dealing with mortalities in hatchery produced oysters but not to the extent previously experienced in 2007 and 2008.

Mr Barton advised he knew of no difficulties in growing hatchery produced oysters being experienced by NT licensees.

Mr Moase confirmed that Clipper had experienced a slightly higher rate of mortalities pre seeding in transport of wildstock oysters last year, in the range of 10% which varied between transports but was unsure if this was related to OOD. Dr Mills confirmed Paspaley had also experienced some increase in mortalities.

Licensees were requested to keep the Department informed about any shell deaths outside the normal range of mortalities.

3 Discussion of Results / Status of OOD PCR Test

Dr Jones informed members that the Micro-array Project and Chlamydiales Project were now completed and the draft final reports had been submitted to the FRDC.

Dr Jones had previously issued a background paper on these two projects to the working group for consideration at this meeting.

Members considered the paper and acknowledged that stress was probably a contributor in causing change in the activity of genes in oysters, switching some on and others off, altering characteristics such as growth rate and susceptibility to disease.

Associate Professor Dr Raftos advised that due to there not being enough DNA sequence, tests could not identify a substantial number of the genes. However the research had allowed establishment of a list of stress response genes affected by particular types of environmental stress. This list could be used to gauge effects of environmental stress during routine husbandry processes on farms.

A/Prof Dr Raftos informed members it was his belief there was a link between stress and the OOD but this had yet to be made scientifically. Given results from other research recently it was also feasible that exposure to air may be a critical component in stress.

Dr Mills reported that Paspaley were endeavouring to assess the stress responses of their oysters in conjunction with OOD.

Dr Jones advised that the Department still held a quantity of frozen samples from 2007 and 2008 of oysters infected with OOD. These samples were still being tested by the Department, as time, money and human resources allowed. Stress tests were also continuing on healthy shell in an attempt to get a result.

A/Prof Dr Raftos explained that a problem with the one factor known to affect Sydney rock oysters ended up being heavy metal contamination; however the biggest environmental issue in the context of QX disease was the rapid changes in pH salinity levels.

Dr Jones advised that more analysis was required on the relationship between Chlamydia and Adenovirus as it had an association with OOD. Dr Jones suggested the two combined might be what is causing the mortalities. More testing however is required.

Members were informed that the Department of Agriculture were commissioning a new electron microscope. The microscope would help immensely in the testing being carried out.

Mr Barton advised that the Northern Territory pearl shell had not been afflicted with OOD.

Dr Jones advised that with the movement of shell between WA and the NT, it was highly likely that the OOD was in the NT but no catalyst had set it off. Something as simple as water temperature could be all it would take.

A/Prof Dr Raftos suggested an alternative approach may be to carry out some analytical work to determine what happens to the oyster under certain conditions and then work back to determine the pathogen.

He also suggested that the best way around OOD may be to check out if there are any genetic groups that are resistant to disease and develop a selective breeding program. NSW may be able to assist WA in this type of research programme.

5 Update on the Pacific Oyster Industry in NSW – similarities with OOD and potential for testing of OOD positive samples

Dr Jones advised that the AAHL were still in the process of producing a positive control to micro-variant oyster herpesvirus which would allow testing of OOD affected oysters.

Dr Jones reported that in 2008, France suffered huge mortalities in their edible oyster industry caused by a micro-variant herpes-virus causing 80% of oysters to die. The micro variant spread through Europe and New Zealand, eventually arriving in Sydney Harbour and Georges River probably via shipping movements. Surveys have reported that aside from two areas in Georges River in Sydney Harbour, Australia is free from the virus. Dr Jones commented that it was interesting that a mutation arose in oyster herpesvirus, which affects many species of bivalve and the mutation (micro-variant) is lethal only to *Crassostrea gigas* (where the disease is known as Pacific Oyster Mortality (POMS)). Dr Jones suggested OOD may have a similar genesis.

The other matter of interest is that co-cultivation of *C. gigas* with other filter feeding molluscs seems to provide protection from POMS and perhaps the same could apply to OOD. While this is noteworthy, tests would need to come from farm observations.

6 Any Other Business

Dr Mills queried how the Department's fish health programmes would be funded under the new fee structure noting that historically, funding had been provided through the cost recovery model. Dr Mills advised of his view that as a core function of government, fish health research should be internally funded.

Dr Jones advised this needed to be clarified formally by government however at present the FRDC was providing funding for ongoing research.

Members discussed the possibility of applying for more funding for research purposes and agreed a future research proposal should be produced.

Action: A/Prof Raftos and Dr Jones to consult and produce a future research programme (including funding requirements) for consideration by the Pearl Oyster Health Working Group.

7 Close

The Chairman declared the meeting closed at 4.10pm.

Cross-Infection Trials of Mystery Condition affecting pearl oysters, Pinctada maxima

Zoe Spiers and Doug Bearham

Aim:

To coinhabit oysters from an affected region with naïve oysters and look for signs of infection in the naïve population.

Materials:

Affected oysters have been supplied from Wapet Shoal and unaffected spat from Point Lefroy. Aquarium facilities at Murdoch University's Fish Health Unit.

Methods:

Cross Infection Trial 1 Methods

Aquariums:

Four Aquariums 18" x 12" x 2' with internal filtration will be used in the experiment.

- Tank 1 was the negative control tank, containing 50 oysters that have not had any exposure to the pathogen, kept at 23ºC
- Tank 2 was the first experimental tank, with 10 affected and 54 unaffected oysters cohabiting in 23ºC.
- Tank 3 was the second experimental tank, with 10 affected oysters cohabiting with 48 unaffected oysters at a higher temperature of 28ºC.
- Tank 4 was the positive control tank, containing 20 affected shell at 23ºC.

Sampling:

All tanks will be sampled every 24 hours from day 5 for the length of the experiment. Any mortalities were recorded, as was the temperatures maintained in the tanks. Oysters will be removed each day, cut in half with half fixed in formalin and the remainder frozen.

Feeding:

Oysters were maintained on daily feeds of nutritional algae, Tahitian *Isochrysis galbana*, *Pavlova lutheri,* and C*haetoceros muelleri*. This also served as a partial daily water change for each of the tanks.

Cross Infection Trial 2 Methods

Aquariums:

Six Aquariums 18" x 12" x 2' with internal filtration will be used in the experiment (Figure 1). The temperature, water used and oysters contained in these tanks are outlined in the table below (Table 1).

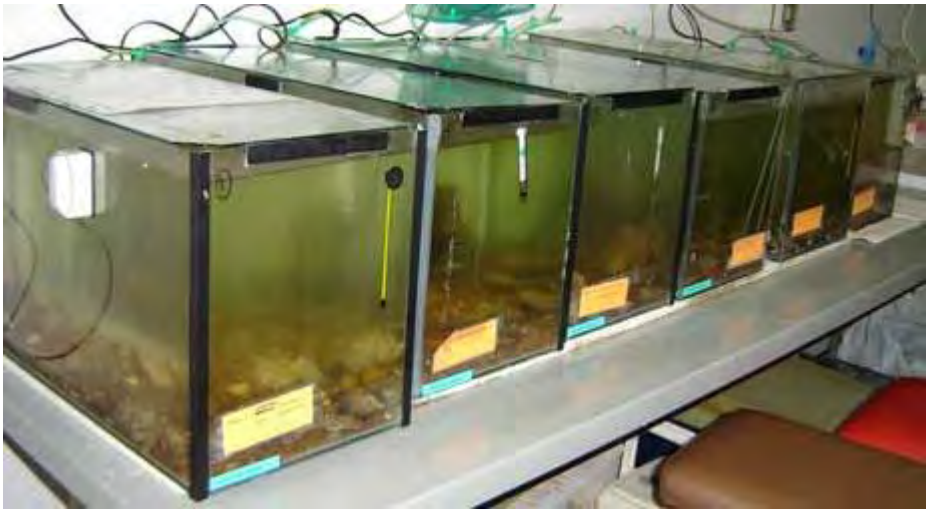

Figure 1: *Photo of the re-circulation tanks used during the Cross Infection Trial 2.*

Table 1: *Conditions in each of the tanks involved in the second cross infection trial.*

Tank	1	2	3	4	5	6
Affected oysters	0	5	7	0	7	8 innoculated spat
Unaffected oysters	25	0	25	25	25	25
Temperature	23ºC	23ºC	23ºC	23ºC	28ºC	23ºC
Water	Aged water + aquarium salt	Aged water + aquarium salt	Aged water + aquarium salt	Water from tank holding affected oysters	Aged water + aquarium salt	Aged water + aquarium salt

Tank 1 was a negative control tank, containing only oysters that have not had any exposure to the pathogen.
Tank 2 was the positive control tank, containing only affected shell at 23ºC.
Tank 3 was the first cross infection tank, with affected and unaffected oysters cohabiting in 23ºC.
Tank 4 contained only unaffected shell, but will be filled with water from the holding tank of affected shell. This is to see whether exposure to water from affected shell also transmits the pathogen.
Tank 5 was the second cross infection tank, with the same conditions as Tank 3 but with a higher temperature of 28ºC.
Tank 6 contained unaffected spat coninhabiting with a few shell that have been experimentally innoculated with concentrated tissue from affected oysters.

Sampling:
Oysters from the tanks were sampled every 24 hours from day 1 for the length of the experiment. Any mortalities were recorded, as was the temperatures maintained in the tanks. Oysters selected for sampling were removed each day with tissue samples fixed in sea-water buffered glutaraldehyde and formalin and/or frozen.

Feeding:
Oysters were maintained on daily feeds of nutritional algae, Tahitian *Isochrysis galbana*, *Pavlova lutheri,* and C*haetoceros muelleri*. This also served as a partial daily water change for each of the tanks.

Results:

Cross Infection Trial 1 Results

Table 2: *Tabled results of observations of temperature and mortalities of the tanks during the trial, and the samples taken.*

Date	Tank 1			Tank 2			Tank 3			Tank 4		
	Temp (ºC)	Deaths	Sampled	Temp (ºC)	Deaths	Sampled	Temp (ºC)	Deaths	Sampled	Temp (ºC)	Deaths	Sampled
14/11 (0)	25	0	2	25	0	2	25	0	2	25	0	3
15/11 (1)	25	0	0	25	0	0	25	3	0	25	0	0
16/11 (2)	25	0	0	24	0	0	25	2	0	25	0	0
17/11 (3)	22	0	0	23	0	0	23	1	0	20	0	0
18/11 (4)	22.5	0	0	22.5	0	0	24	1	0	23	0	0
19/11 (5)	22	0	2	22	0	2	25.5	1	2	24	0	0
20/11 (6)	22	0	3	22	0	3	29	1	3	25	0	0
21/11 (7)	22	0	3	22	0	3	28	3	3	24	0	0
22/11 (8)	23	0	3	23	0	3	29	10	3	24	0	0
23/11 (9)	22.5	0	3	23	5	3	29	5	3	23	0	0
24/11 (10)	23	0	3	23	7	3	29.5	2	3	23.5	0	0
25/11 (11)	23	0	3	23	5	3	29	-	-	23	0	0
26/11 (12)	23	0	3	23	1	3	-	-	-	23	0	0
27/11 (13)	22	0	3	23	0	3	-	-	-	23	0	0
28/11 (14)	22	0	3	21	1	3	-	-	-	24	1	0
29/11 (15)	21.5	0	3	21	1	3	-	-	-	23.5	0	0

Tank 1: Negative control
Tank 1 contained 50 unaffected oysters. There were no mortalities in this tank during the course of the 15 day experiment. The temperature in the tank was held between 21.5 and 25ºC. On day 1, two oysters were sampled, on day 5, two oysters were sampled and then 3 oysters were sampled on each consecutive day. A total of 34 oysters were sampled during the experiment.

Tank 2: Cool cross-infection
Tank 2 contained 10 affected and 54 unaffected oysters cohabiting in temperatures between 21 and 25ºC. On day 1, two oysters were sampled, on day 5, two oysters were sampled and then 3 oysters were sampled on each consecutive day. A total of 34 oysters were sampled during the trial. There were no mortalities until day 9 of the trial, when 5 oysters were found moribund. The following days resulted in 7 mortalities, 5, 1, none, 1 and then 1 mortality, bringing the total number of dead oysters to 20 in tank 2.

Tank 3: Warm cross-infection
Tank 3 contained 10 affected oysters cohabiting with 48 unaffected oysters. The temperature started at between 23-25.5ºC then was increased to 28-29.5ºC. On day 1, two oysters were sampled, on day 5, two oysters were sampled and then 3 oysters were sampled on each consecutive day until day 10. A total of 19 oysters were sampled during the trial. Mortalities in this tank began on day 1 and continued until there were no previously unaffected oysters alive by day 10, with a total of 29 mortalities.

Tank 4: Positive control
Tank 4 contained 15 affected oysters with temperatures between 20-25ºC. Three oysters were sampled on day 1, and there was a single mortality occurring on day 14.

Clinical signs
The clinical signs associated with this illness were observed in the warm cross infection tank (Tank 3) on day 4 of the trial, and later in the cool water tank (Tank 2). When observing the spat in the aquarium, the shell was gaping with the mantle pale and withdrawn. However, the gills were still prominent and easily visible as seen in Figure 3. The spat displaying these signs were often sluggish and slow to close when stimulated. In many cases, spat taken from the water because they were thought to be dead and left on the bench, would sometimes snap shut after up to 10 minutes out of the water. Even prodding the adductor muscle with a blunt instrument wouldn't produce an immediate response, however a few minutes later the oyster would suddenly snap closed. The result of this meant spat that were taken out of the aquarium as 'dead' had to be left on the bench for over 10 minutes before they were finally confirmed dead for the purposes of this experiment. When opened, the oysters appeared watery, with prominent gills and a mildly swollen gut (Figure 2).

Figure 2: *Photo of an oyster from the affected area showing clinical signs of the Mystery Condition. The mantle is withdrawn and pale, however the body of the oyster appears healthy in comparison with a mildly swollen gut.*

Figure 3: *Photo of an oyster from the affected area displaying the typical clinical signs associated with the Mystery Condition. The mantle is withdrawn, however the gills are prominent and appear healthy, which is highly unusual for a sick oyster.*

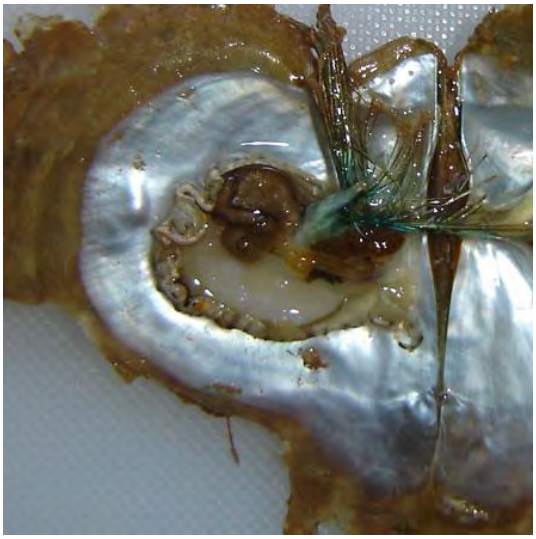

Figure 4

Figure 5

Figure 4: *Photo of a spat from the transmission trial displaying clinical signs of the disease.* **Figure 5:** *Photo of a spat showing typical signs assciated with ill heath, not related to the Mystery Condition. Note the difference in health of the body of the oyster in comparison to the mantle. In the oyster with the Mystery Condition, the mantle is pale and withdrawn however the rest of the oyster appears healthy, whereas in a typically sick oyster, the whole body is affected in addition to the mantle tissue. In typically ill oysters, the gills and body of the oyster appear watery, discoloured and begin to lose their structure, as seen in Figure 5.*

Mortalities

The mortalities in the warm cross infection tank began immediately, reaching their peak on day 8 of the trial. The cooler tank didn't suffer any mortalities until day 9, with the number of deaths increasing on day 10, then subsiding.

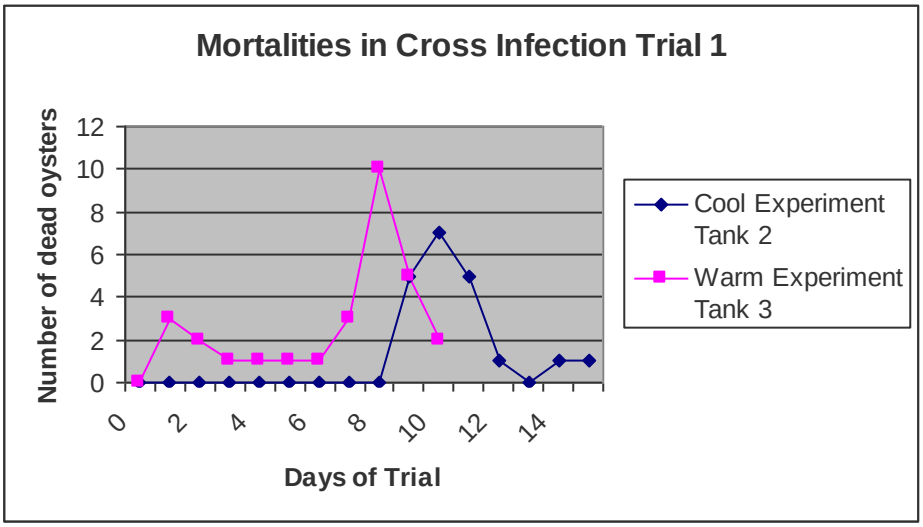

Figure 6: *Graph of mortalities sustained in the experiment tanks during the course of the trial.*

Histology and Electron Microscopy

The histology and electron microscopy for these trials is still pending, however preliminary results from the first trial indicate very similar histological signs to the original martality event.

Cross Infection Trial 2 Results

Tank 1: Negative control

The negative control tank contained 25 unaffected spat. The temperature was held at 21-23ºC during the trial. Oysters were sampled periodically and placed in formalin, glutaraldehyde or frozen. No mortalities were observed.

Tank 2: Positive control

The positive control tank contained 5 oysters from an affected area at between 21-22ºC. One oyster was sampled on day 3, 5 and again on day 14. No mortalities were observed during the trial.

Tank 3: Cool cross-infection

Tank 3 contained 25 unaffected oysters and 7 affected oysters. The temperature was kept at 21-24ºC during the experiment, and oysters were sampled daily until no oysters remained alive. On day 3, one affected oyster died, followed by two on day 7 and finally one on day 14, totalling 4 dead affected oysters. Of the unaffected population, no mortalities were recorded until day 7 where one died, then two on day 8, one on day 11 and finally two on day 14. A total of 6 unaffected oysters died. The rest of the population was collected for sampling.

Tank 4: Water from the holding tank for affected oysters

Water for this tank was sourced from the tank used to hold the affected oysters. 25 unaffected oysters were placed in the tank and held between 22-24ºC. Oysters were selected for sampling every day and apart from these, no other mortalities were observed for the length of the trial.

Tank 5: Warm cross-infection
This tank was held at 28.5ºC and contained 7 affected oysters and 25 unaffected oysters. Mortalities began on day 3 where one unaffected oyster died, followed by 5 affected and 4 unaffected on day 4 and then all remaining oysters were found dead on day 5. A total of 21 unaffected oysters died.

Tank 6: Innoculated tank
This tank contained 25 unaffected oysters and 8 previously-unaffefcted oysters that were bathed in a solution made from the tissue of affected oysters. Three of these 'innoculated' oysters were sampled on day 1 and the remaining died on day 5. Of the unaffected population, the first mortality was on day 6, followed by 4 on day 8, one on day 9 and they were all deceased by day 10.

It should be noted that a power failure due to a broken overhead power line occurred on day 12 of the trial. This caused the airconditioner in the room to be off along with all the filters and aerators. The time the power was out was less than 24 hours.

Clinical signs
The first obvious clinical signs were observed in Tank 3 and Tank 6 on day 6 of the trial, where the oysters were sluggish and had withdrawn, pale mantle.

Histology and Electron Microscopy
The histology and electron microscopy for the second trial is still pending. Until there is histological substantiation, it cannot be confirmed that the 'affected' oysters used in the Cross infection Trial 2 were in fact affected with the Mystery Condition. Since the affected oysters that were used in Trial 2 were the survivors of the Trial 1, these may have been the less-affected, recovered or immune oysters of the affected population.

Table 3: *The data collected during the second cross infection trial. The mortalities are all unaffected spat unless otherwise stated.*

Date	Tank 1 (+ve control)			Tank 2 (-ve control)			Tank 3 (Cool x-infn)			Tank 4 (+ve water)			Tank 5 (Hot x-infn)			Tank 6 (Innoculatd)		
	Temp ºC	Deaths	Samples	Temp ºC	Deaths	Samples	Temp ºC	Deaths	Samples	Temp ºC	Deaths	Samples	Temp ºC	Deaths	Samples	Temp ºC	Deaths	Samples
14 Dec (1)	23	0	G: F: I:	22	0	G: F: I:	24	0	G: F: I:	23.5	0	G: F: I:	23	0	G: F: I:	24	0	G: F:X I:X
15 Dec (2)	21	0	G:X F: X I:	21	0	G: F: I:	22	0	G:X F:X I:	24	0	G:X F:X I:	28.5	0	G:X F:X I:	23	0	G:X F:X I:
16 Dec (3)	21	0	G:X F:X I:X	21	0	G: F:X I:X	22	1 Aff	G:X F:X I:X	24	0	G:X F:X I:X	28.5	1	G:X F:X I:X	22.5	0	G:X F:X I:X
17 Dec (4)	22	0	G: F: I:	21	0	G: F: I:	22	0	G:X F:X I:	24	0	G:X F:X I:	28.5	5 Aff	G:X F:X I:	23	0	G:X F: I:
18 Dec (5)	22	0	G: F:X I:X	21.5	0	G: F:X I:X	22	0	G: F:X I:X	24	0	G: F:X I:X	-	-	G: F: I:	23	5 inn	G: F:X I:X
19 Dec (6)	22	0	G: F:X I:X	21.5	0	G: F: I:	22	0	G: F:X I:X	24	0	G: F:X I:X	-	-	G: F: I:	23	1	G: F:X I:X
20 Dec (7)	21.5	0	G:X F:X I:	21	0	G: F: I:	23	2 Aff 1 Unaf	G:X F:X I:	22	0	G:X F:X I:	-	-	G: F: I:	22.5	0	G:X F:X I:
21 Dec (8)	21.5	0	G:X F:X I:	21	0	G: F: I:	22	2	G:X F:X I:	22	0	G:X F:X I:	-	-	G: F: I:	23	4	G:X F:X I:

Table 3 *continued…*

Date	Tank 1			Tank 2			Tank 3			Tank 4			Tank 5			Tank 6		
	Temp	Deaths	Samples	Temp	Deaths	Samples	Temp	Deaths	Samples	Temp	Deaths	Samples	Temp	Deaths	Samples	Temp	Deaths	Samples
22 Dec (9)	21.5	0	G:X F:X I:	21	0	G: F: I:	21.5	0	G:X F:X I:	22.5	0	G:X F:X I:	-	-	G: F: I:	23	1	G:X F:X I:
23 Dec (10)	21.5	0	G:X F:X I:	21	0	G: F: I:	21.5	0	G:X F:X I:	22	0	G:X F:X I:	-	-	G: F: I:	22.5	-	G:X F:X I:
24 Dec (11)	21.5	0	G:X F:X I:	21	0	G: F: I:	21.5	1	G:X F:X I:	22	0	G:X F:X I:	-	-	G: F: I:	-	-	G: F: I:
25 Dec (12)	22	0	G: F:X I:	21.5	0	G: F: I:	22	0	G:X F:X I:	22	0	G: F:X I:	-	-	G: F: I:	-	-	G: F: I:
26 Dec (13)	23	0	G: F:X I:	22	0	G: F: I:	23	0	G: F:X I:	23	0	G:X F:X I:	-	-	G: F: I:	-	-	G: F: I:
27 Dec (14)	21.5	0	G: F:X I:	21	0	G: F:X I:	22	1 Aff 2 Unaf	G:X F:X I:	22	0	G: F:X I:	-	-	G: F: I:	-	-	G: F: I:
28 Dec (15)	22	0	G: F:X I:	21.5	0	G: F: I:	22	-	G: F: I:	22	0	G: F:X I:	-	-	G: F: I:	-	-	G: F: I:

G: sea-water buffered 5% glutaraldehyde, F: 10% formaldehyde, I: frozen samples. ‘X’ indicating types of samples obtained.

REFERENCES

Anderson DR, Hopps HE, Barile MF, Bernheim BC (1965) Comparison of the Ultrastructure of Several Rickettsiae, Ornithosis Virus, and *Mycoplasma* in Tissue Culture. *Journal of Bacteriology* **90**, 1387-1404.

Barzon L, Lavezzo E, Costanzi G*, et al.* (2013) Next-generation sequencing technologies in diagnostic virology. *Journal of Clinical Virology* **58**, 346-350.

Barzon L, Lavezzo E, Militello V, Toppo S, Palù G (2011) Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences* **12**, 7861.

Beatty WL, Morrison RP, Byrne GI (1995) Reactivation of persistent *Chlamydia trachomatis* infection in cell culture. *Infection and Immunity* **63**, 199-205.

Biberfeld G, Biberfeld P (1970) Ultrastructural features of Mycoplasma pneumoniae. *Journal of Bacteriology* **102**, 855-861.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*.

Comps M, Tigé G (1999) Procaryotic infections in the mussel *Mytilus galloprovinciallis* and in its parasite the turbellarian *Urastoma cyprinae*. *Diseases of Aquatic Organisms* **38**, 211-217.

Corbeil S, Colling A, Williams LM*, et al.* (2010) Development and validation of a TaqMan PCR assay for the Australian abalone herpes-like virus. *Diseases of Aquatic Organisms* **92**, 1-10.

Crockford M, Jones B (2012) Aquatic Animal Health Subprogram: Investigation of Chlamydiales-like organisms in pearl oysters, *Pinctada maxima*, pp. 1-40. Final Report FRDC Project No. 2008/031. Fisheries Research Report No. 232. Department of Fisheries, Western Australia.

Davidson NM, Oshlack A (2014) Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biology* **15**, 410.

Epstein JH, Quan P-L, Briese T*, et al.* (2010) Identification of GBV-D, a Novel GB-like Flavivirus from Old World Frugivorous Bats (*Pteropus giganteus*) in Bangladesh. *PLoS Pathogens* **6**, e1000972.

Gómez-Chiarri M, Guo X, Tanguy A, He Y, Proestou D (2015) The use of -omic tools in the study of disease processes in marine bivalve mollusks. *Journal of Invertebrate Pathology* **131**, 137-154.

Humphrey JD, Barton MA (2009) An unsuccessful attempt to experimentally transmit "oyster oedema disease syndrome" to pearl oyster spat. In: *Technote*, pp. 1-7, Darwin.

Humphrey JD, Norton JH, Jones JB*, et al.* (1998) Pearl oyster (*Pinctada maxima*) aquaculture: health survey of Northern Territory, Western Australia and Queensland pearl oyster beds and farms. FRDC Final Report - PROJECT No. 94/079.

Jones JB (2008) The Australian experience: pearl oyster mortalities and disease problems. In: *Pearl Oyster Health Management: A Manual* (ed. Bondad-Reantaso M MS, Berthe FCJ), pp. 87–93. FAO Fisheries Technical Paper, Rome.

Jones JB (2012) Minutes Pearl Oyster Health Working Group Meeting. Monday 20 February 2012. see Appendix 9.

Jones JB, Crockford M, Creeper J, Stephens F (2010) Histopathology of oedema in pearl oysters *Pinctada maxima*. *Diseases of Aquatic Organisms* **91**, 67-73.

Jones JB, Raftos DA (2012) Oyster Oedema Disease - Research Priorities. Research priorities document for the Australian pearl oyster industry prepared for the Pearl Oyster Health Working Group. see Appendix 9.

Kuo CC, Chi EY (1987) Ultrastructural study of *Chlamydia trachomatis* surface antigens by immunogold staining with monoclonal antibodies. *Infection and Immunity* **55**, 1324-1328.

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**, 357-359.

Lecuit M, Eloit M (2015) The potential of whole genome NGS for infectious disease diagnosis. *Expert Review of Molecular Diagnostics* **15**, 1517-1519.

Li D, Wu X (2004) Purification and biological features of a rickettsia-like prokaryote from the scallop *Argopecten irradians* in China. *Aquaculture* **234**, 29-40.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.

Løvoll M, Wiik-Nielsen J, Grove S*, et al.* (2010) A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS). *Virology Journal* **7**, 309.

Madin B (2007) An investigation into a pearl oyster (*Pinctada maxima*) mortality event, pp. 1-29. Report prepared for the Department of Fisheries, Western Australia by AusVet Animal Health Services Pty Ltd. see Appendix 9.

Mardis ER (2008) Next-generation DNA sequencing methods. *Annual Review of Genomics and Human Genetics* **9**, 387-402.

Molloy DP, Giamberini L, Morado JF, Fokin SI, Laruelle F (2001) Characterization of intracytoplasmic prokaryote infections in Dreissena sp. (Bivalvia: Dreissenidae). *Diseases of Aquatic Organisms* **44**, 203-216.

Moore RA, Warren RL, Freeman JD*, et al.* (2011) The sensitivity of massively parallel sequencing for detecting candidate infectious agents associated with human tissue. *PLoS One* **6**, e19838.

Moulder JW (1991) Interaction of chlamydiae and host cells in vitro. *Microbiology Reviews* **55**, 143-190.

Pepin JF, Riou A, Renault T (2008) Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *Journal of Virological Methods* **149**, 269-276.

Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews* **62**, 1094-1156.

Renault T, Cochennec N (1995) Chlamydia-like organisms in ctenidia and mantle cells of the Japanese oyster *Crassostrea ggias* from the French Atlantic coast. *Diseases of Aquatic Organisms* **23**, 153-159.

Savage J, Hobsbawn P (2015) Australian fisheries and aquaculture statistics 2014. In: *Fisheries Research and Development Corporation project 2014/245*. Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES), Canberra.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols* **3**, 1101-1108.

Spiers Z, Bearham D (2006) Cross-infection trials of mystery condition affecting pearl oysters, *Pinctada maxima*. Project 2013/002, pp. 1-27. Printed: 14 May 2013. see Appendix 9.

Sun J, Wu X (2004) Histology, ultrastructure, and morphogenesis of a rickettsia-like organism causing disease in the oyster, *Crassostrea ariakensis* Gould. *Journal of Invertebrate Pathology* **86**, 77-86.

Wilson MH, Collier AM (1976) Ultrastructural study of Mycoplasma pneumoniae in organ culture. *Journal of Bacteriology* **125**, 332-339.

Wu X, Li D, Pan J (2001) Studies on Rickettsia-like organism disease of the tropical marine pearl oyster–epidemiological investigation of RLO disease in larval populations of maricultured *Pinctada maxima*. *Acta Oceanologica Sinica* **20**, 563-574.

Wu X, Pan J (1999) Studies on Rickettsia–like Organism Disease of the Tropical Marine Pearl Oyster I: The Fine Structure and Morphogenesis of *Pinctada maxima* Pathogen Rickettsia–like Organism. *Journal of Invertebrate Pathology* **73**, 162-172.

Xie F, Xiao P, Chen D, Xu L, Zhang B (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology* **80**, 75-84.